Oestrogen regulation of gene expression in male germ cells and Sertoli cells

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For my family

Declaration

The studies undertaken in this thesis were the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification

Sharon Sneddon
October 2004

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Abstract

The testis has two main functions, the synthesis of steroid hormones and the production of spermatozoa. The adult testis contains three main somatic cell types, namely Sertoli cells, Leydig cells and peritubular myoid cells, as well as germ cells at all stages of maturation. Interactions between these cells and the steroid hormones produced by the testis are responsible for the regulation and maintenance of spermatogenesis and fertility. Depletion or exposure to high levels of oestrogens, or androgens, both have an adverse impact on male reproductive function. In the testis, as well as in other organs, steroid hormone action is mediated by ligand-activated receptors. A single androgen receptor (AR) and two oestrogen receptors (ERα and ERβ) have been identified.

The aims of this study were to investigate the role of steroid hormones, in particular oestrogens, in murine spermatogenesis. A major focus of these investigations was the role played by ERβ in the modulation of germ cell and somatic cell function. Studies were conducted both using a transformed murine Sertoli cell line (SK11), which has maintained a differentiated Sertoli cell phenotype and spermatogonial stem cells, which were successfully isolated and characterised. Steroid hormone receptor status, steroid responsiveness and the impact of targeted deletion using RNAi were all assessed.

Characterisation of the SK11 cell line, which was cultivated under conditions which maintained them in an undifferentiated or differentiated state, revealed they retain many features of Sertoli cells *in vivo*. ERβ mRNA and protein were shown to be expressed in the SK11 cells both in the undifferentiated and differentiated states. Transient transfections using ERE or ARE-luciferase reporter constructs and stimulation with steroid ligands revealed that the cells contained functional steroid hormone receptors. Knockdown of ERβ mRNA and protein was achieved in the cells after targeted deletion using a short hairpin RNAi containing vector; this blunted the ability of the cells to respond to oestrogen.

Isolation of spermatogonial stem cells was carried out using immunomagnetic beads. The stem cell population were shown to express Oct-4 and GFRα-1 mRNAs, both of which are stem cell markers, but not c-kit, which is a marker of differentiated germ cells. Taqman Q-RT-PCR demonstrated that the stem cell population expressed ERβ. Oct-4 mRNA expression was shown to be reduced by RNAi; this induced the cells to undergo differentiation *in vitro* characterised by increased expression of c-kit.

In conclusion, the current studies have extended our understanding of the impact of steroid hormones on testicular function and have revealed for the first time that spermatogonial stem cells are ERβ positive. The SK11 cell line has been found to provide a suitable model system for the study of steroid regulation of Sertoli cell function. In addition, the use of RNAi has provided an exciting new avenue by which to manipulate gene expression levels in testicular germ and somatic cells.

Presentations relating to this thesis

ERß expression in a transformed Sertoli cell line (SK11)

Presented at the 13th European Testis Workshop, Dunblane Scotland, April 2004

Oestrogen receptor β expression in spermatogonial stem cells

Poster presented at the International Congress of Endocrinology, Lisbon, September 2004.

Isolation, characterisation and *in vitro* differentiation of murine spermatogonial stem cells

Presented at the MRC Eggnet meeting, October 2004

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Abbreviations

ABC	Avidin biotin complex
AR	Androgen receptor
bp	Base pairs
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cAMP	Cyclic AMP
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
CsCl	Caesium chloride
Ct	Threshold cycle
DAB	Diaminobenzadine
dH_20	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DMEM	Dulbeccos modified Eagles media
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
EGFP	Enhanced green fluorescent protein
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
EtOH	Ethanol
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
FITC	Fluorescein isothiocyanate conjugate
GnRH	Gonadotrophin releasing hormone
HRP	Horse radish peroxidase
kb	Kilobase
kDa	Kilodalton
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride

NaOH	Sodium hydroxide
nt	Nucleotide
OD	Optical density
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PGC	Primordial germ cell
Q RT-PCR	Quantitative reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	RNA interference
rpm	Revolutions per minute
RT	Reverse transcription
SAP	Shrimp alkaline phosphatase
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
TBS	Tris buffered saline
v/v	Volume to volume ratio
w/v	Weight to volume ratio

Chapter One

Literature review

1.1. Introduction

In the adult testis, the process of spermatogenesis is controlled by a complex system of endocrine and paracrine signals acting upon, and within the seminiferous epithelium. The testis is unusual in that it is the only organ in the adult, which contains cells undergoing meiosis. The testis determines the phenotype of an individual based on its endocrine activity (Schlatt et al., 1997) and it is the steroid hormones that are key regulators of spermatogenesis and male fertility (Sharpe, 1994).

Spermatogenesis is an important process, as without the production of spermatozoa, genetic information would not be passed on (Sharpe, 1994). The testis contains totipotent stem cells, which continually repopulate the testis with germ cells. Approximately 50% of human infertility is due to problems with the male and 70-90% of these cases are due to disruption of spermatogenesis. Better understanding of the process of spermatogenesis and its regulation by steroid hormones could lead to increased knowledge about the formation of testicular tumours, ways to improve treatments for male infertility, and perhaps provide methodology to produce novel male contraceptives.

1.2. General organisation of the testis

The testis has two main functions, the production of spermatozoa and the production of hormones (Schlatt et al., 1997). The adult testis contains two distinct compartments, the seminiferous tubules, which contain the Sertoli cells and the germ cells (Figure 1.1) and the interstitium, which comprises of the Leydig cells, peritubular myoid cells, macrophages and blood vessels. These compartments are structurally and physiologically distinct, separated by cellular barriers, which limit the exchange of water-soluble materials (Sharpe, 1994).

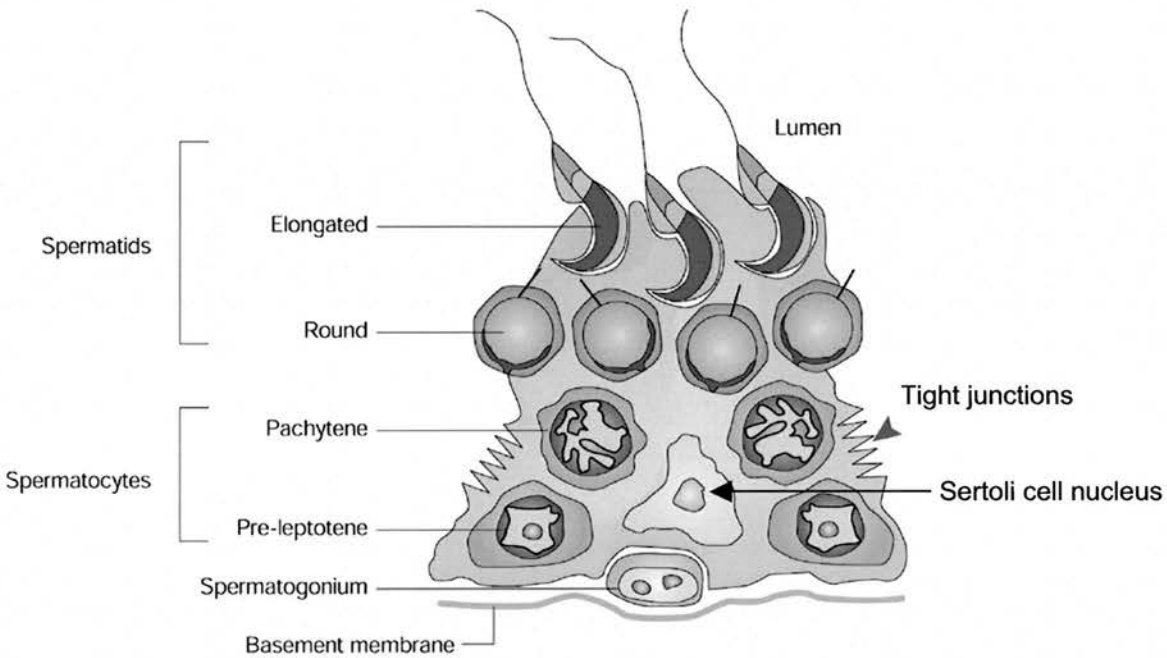


Figure 1.1 Diagram of a Sertoli cell with its associated germ cells. Spermatogonia and spermatocytes are situated in the basal compartment of the testis while the more mature germ cells; the spermatids and the spermatozoa are found in the adluminal compartment. The two compartments are separated by the tight junctions of the Sertoli cell. Adapted from Cooke and Saunders (2002).

Differentiating germ cells are not arranged at random in the seminiferous epithelium (Sharpe, 1994), but in specific associations known as "stages". The cycle of the seminiferous epithelium was first defined in the rat by Leblond and Clermont (Leblond and Clermont, 1952). They defined 14 stages in the rat cycle based on the 19 phases of spermiogenesis as identified by periodic acid Schiff (PAS) staining. A stage is defined as a grouping of germ cell types at particular phases of development in cross sectioned tubules (Russell et al., 1990). The 1994 review by Sharpe describes in detail, the spermatogenic cycle of various species. The cycle charts the progress of a single spermatogonia through successive mitoses, meiosis, and spermiogenesis, finishing with the release of a mature spermatozoa. Initiation of spermatogenesis is cyclical. The cycle of the seminiferous epithelium lasts approximately 16 days in man and 8.6 days in the mouse, therefore 4.6 and 4.06 cycles are required from the time a spermatogonium becomes committed to

differentiation until it is released from the seminiferous epithelium as a mature spermatozoa in the human and the mouse respectively (Sharpe, 1994). Figure 1.2 shows how the germ cells are associated at different stages of the cycle in the mouse.

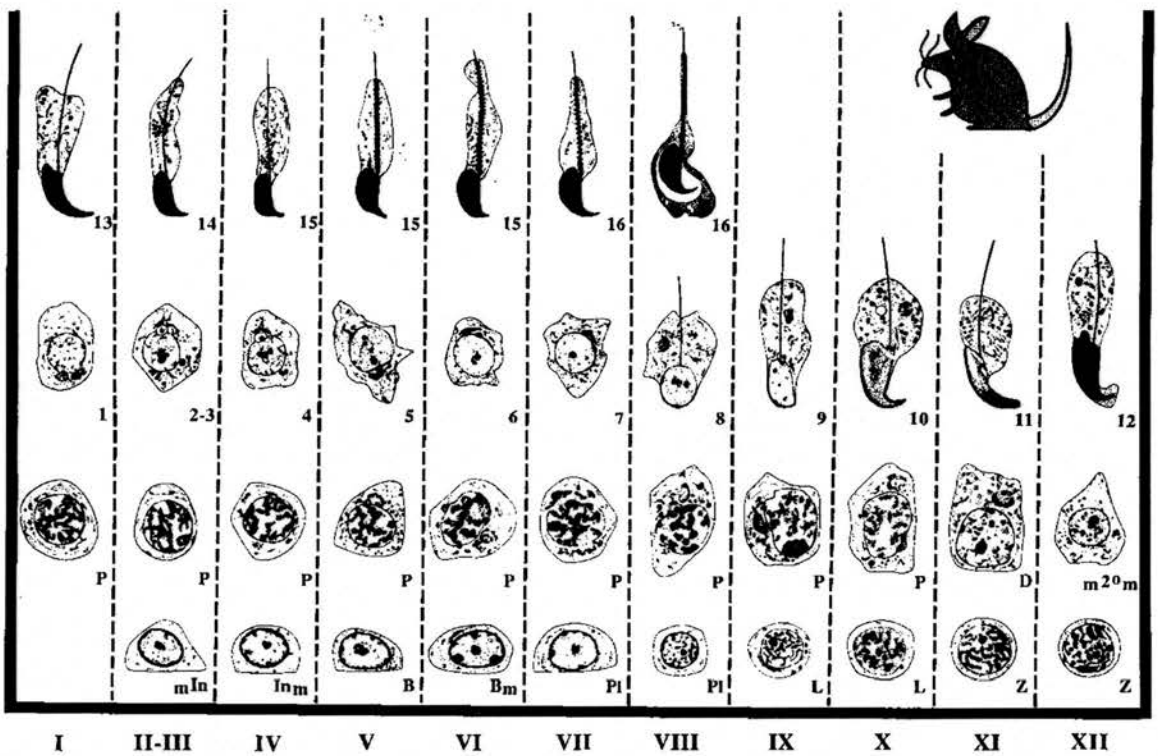


Figure 1.2 Spermatogenic cycle in the mouse. Taken from Russell et al. (1990).

Each vertical column represents a stage; each stage has a variety of germ cells associated with the Sertoli cells. Each stage lasts for a fixed period of time and the germ cells move from one stage to the next. In the diagram shown in Figure 1.2, germ cells progress in stages from left to right. Once the germ cells reached the final stage they progress back to stage I again, but this time are one row up (Sharpe, 1994).

1.2.1. Seminiferous epithelium

Germ cell maturation occurs within the seminiferous epithelium which is organised into long tubules, maximising the area for sperm production. Close morphological

associations between Sertoli cells and germ cells at different stages of their development (spermatogonia, spermatocytes, round spermatids, and elongated spermatids) exist (Figure 1.1) and as a result of these associations, extensive interactions and communications take place between these cells throughout spermatogenesis both at the biochemical and molecular level (Cheng and Mruk, 2002). Within the seminiferous tubules, immature germ cells are positioned on the basement membrane. As the germ cells differentiate, they move through the tubule towards the apical surface gradually maturing before being released into the tubule lumen as mature spermatozoa. This results in the seminiferous epithelium containing several layers of germ cells at different stages of development at any one time (Sharpe, 1994).

Russell demonstrated the progressive movement of primary spermatocytes from the basal to the adluminal compartment of the seminiferous tubule during investigations into the integrity of the seminiferous epithelium. He concluded that the Sertoli cells play an active role in the transfer of spermatocytes to the adluminal compartment and suggested that a transient intermediate compartment existed within the seminiferous tubule, which allowed the continual maintenance of the blood-testis barrier during transit of spermatocytes from the basal to the adluminal compartment (Russell, 1977).

Chiarini-Garcia et al. (2001) reported that spermatogenesis in the mouse occurs in a uniform pattern and the spermatogonia are distributed in a non-random manner (Chiarini-Garcia et al., 2001). They showed that spermatogonia were spaced throughout the seminiferous epithelium in such a way that the initiation of spermatogenesis is evenly distributed. The same group also demonstrated that in the mouse, extensive tubule-to-tubule contact occurred with three to seven tubules being in contact within the seminiferous epithelium. In the rat, the positioning of the spermatogonia is similar but there is less tubule–to-tubule contact seen (Chiarini-Garcia et al., 2003). There is a fixed germ cell to Sertoli cell ratio within the testis. This varies between species (Sharpe, 1994) and results in the widely differing spermatozoa outputs in different species.

1.2.2. Interstitium

The seminiferous tubules are separated from one another by the interstitium of the testis. This region contains the hormone secreting Leydig cells and the peritubular myoid cells as well as macrophages, blood vessels and endothelial cells (Saez, 1994). The endocrine function of the testis lies primarily within the Leydig cells, which are heterogeneous in respect to their physiological and structural features (Schlatt et al., 1997). In the human, they are steroidogenically active in the period of early differentiation of the male embryo. In the rodent, there is a surge in the levels of the pituitary gonadotrophin luteinising hormone (LH) and testosterone at about embryonic day 20 (Chemes et al., 1979). At puberty, gonadotrophin release from the pituitary stimulates androgen secretion by the Leydig cells (Haider, 2004). Similarly, in the mouse, two populations of Leydig cells exist. The first, a fetal population, is seen at about 12.5 days postcoitum, soon after testis differentiation. This population is essential for masculinisation of the fetus. The second population is found after birth in the mouse. These cells are the testosterone secreting population and they act to induce male behaviour and secondary sexual characteristics (Baker and O'Shaughnessy, 2001; O'Shaughnessy et al., 2002).

Testosterone biosynthesis is under the control of LH. LH stimulates the production of cyclic AMP (cAMP) by binding to receptors on the Leydig cell surface (Cooke et al., 1981). cAMP has two principal activities in the control of Leydig cell steroidogenesis: The first action of cAMP is the stimulation of testosterone biosynthesis via the transport of cholesterol into the steroidogenic pathway. The second action of cAMP in Leydig cells is the stimulation of gene expression of the steroidogenic enzymes and up-regulation of their activity (Stocco and Clark, 1996). Steroid biosynthesis is described in more detail in section 1.7.

Also present in the interstitium of the testis is a layer of contractile peritubular myoid cells, which surround the tubules. These cells are modified myofibroblast cells and they lie external to the basement membrane of the seminiferous tubule. These cells do not provide a tight diffusion barrier into the tubule but they are responsible for the

irregular contractions of the seminiferous tubules which propel fluid secreted by the Sertoli cells, together with the spermatids into the lumen and through the tubule to the rete testis (Clermont, 1958; Schlatt et al., 1997). Peritubular myoid cells also maintain mesenchymal-epithelial interactions with the Sertoli cells by deposition of extracellular matrix elements (Verhoeven et al., 2000), this separates the testicular cords from the interstitial compartment (Martineau et al., 1997; Tilmann and Capel, 1999; Tung et al., 1984). The peritubular myoid cells express androgen receptor (Zhou et al., 2002) and produce PmodS (Peritubular factor that Modulates Sertoli cell function). PmodS production is stimulated by androgens and is involved in the regulation of androgen mediated Sertoli cell functions (Norton and Skinner, 1989; Verhoeven et al., 2000). Vascular smooth muscle cells, endothelial cells and macrophages are also present in the interstitial space of the testis.

1.3. Germ cells

Spermatogonial stem cells originate from primordial germ cells (PGCs) derived from the epiblast of the embryo. During fetal development, the PGCs migrate to the genital ridges and in the case of the male, become enclosed by the differentiating Sertoli cells and the seminiferous cords form. Once in the cords, PGCs are termed gonocytes and are morphologically different from the PGCs. Gonocytes then undergo a period of proliferation before becoming quiescent (Clermont and Perey, 1957; Huckins and Clermont, 1968). In the mouse and the rat, the gonocytes differentiate shortly after birth and give rise to the spermatogonial stem cells (Bellve et al., 1977; Clermont and Perey, 1957; Huckins and Clermont, 1968). The production of mature sperm from a single spermatogonia takes approximately 75 days in man and approximately 35 days in mice (Cooke and Saunders, 2002).

Spermatogenesis begins with a series of mitotic divisions of the spermatogonia (Figure 1.3) and the spermatogenic process continues throughout the lifespan of the male. Upon division, spermatogonial stem cells can give 2 types of daughter cells. One of these cells is capable of self-renewal and maintains the stem cell population while the other cell type becomes the type A spermatogonia (de Rooij, 2001; de Rooij and Russell, 2000). The dual capacity of the stem cells ensures the long-

standing ability to produce spermatozoa. Most of our understanding of the development of spermatogenesis comes from detailed studies in rodents. The spermatogonial stem cells (A_s) are located on the basement membrane of the seminiferous tubule and will be further discussed in section 1.6. According to the system proposed by Oakberg (Oakberg, 1971), the non self-renewing cell produced by the division of the spermatogonial stem cell divides into a pair of cells that stay connected by an intercellular bridge (A_{pr} spermatogonia). Although cytokinesis is incomplete within these cells, nuclear division has occured. The cells then further divide to form chains of A-aligned (A_{al} spermatogonia). It is thought that the A_s, A_{pr} and A_{al} spermatogonia are morphologically similar and have similar patterns of regulation (De Rooij and Grootegoed, 1998). These cells undergo a change in cell cycle and differentiate to become the A1 spermatogonia (Chiarini-Garcia and Russell, 2001). The A_s, A_{pr} and A_{al} spermatogonia cycle at random but the A1 and subsequent generations of spermatogonia proliferate at a fixed rate. In the mouse, there are around 10 spermatogonial divisions between stem cells and the formation of spermatocytes (de Rooij and Russell, 2000), as shown in Figure 1.3.

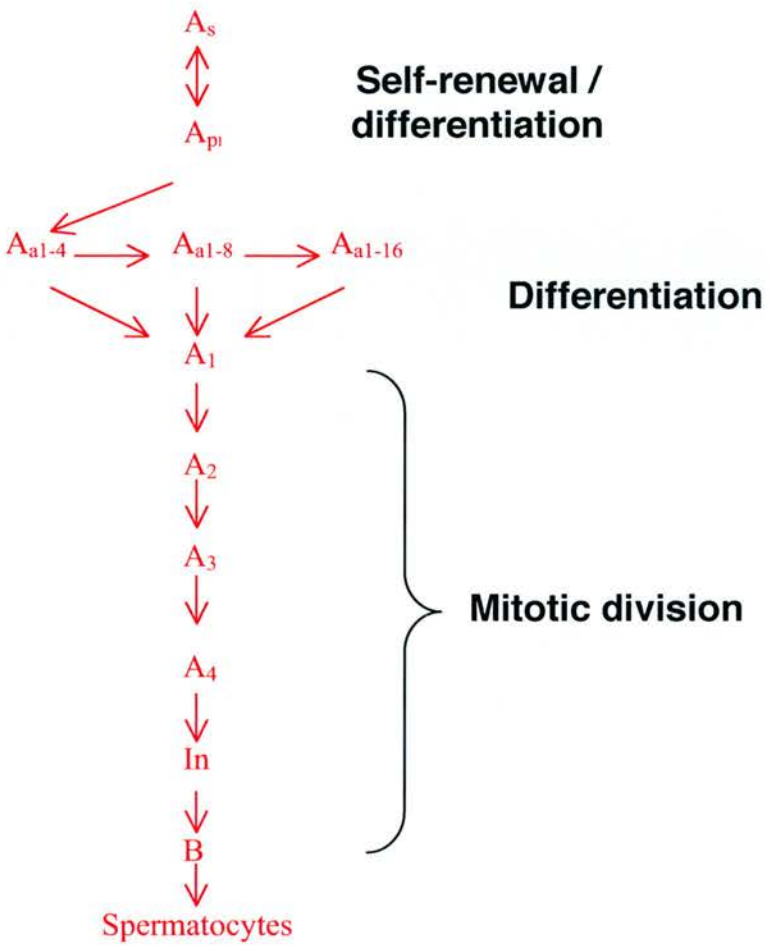


Figure 1.3 Stem cell renewal and spermatogonial differentiation in the mouse. The scheme begins with the spermatogonial stem cell which can either self renew or differentiate to produce A_{pr} spermatogonia, which will continue mitotically dividing until the spermatocytes are formed. Adapted from de Rooij, 2001.

In primates the undifferentiated spermatogonia can be subdivided into A pale (Ap) and A dark (Ad) spermatogonia. Both Ap and Ad are arranged in clones of 1 or 2n cells within the seminiferous epithelium. The Ad spermatogonia do not proliferate, but if cell loss occurs in the testis, they can transform into Ap spermatogonia that start to proliferate. The Ap spermatogonia only divide once every epithelial cycle, undergoing self-renewal and differentiation into B spermatogonia (De Rooij et al., 1989).

The proliferative phase of spermatogenesis takes place in the basal intratubular compartment of the testis. This process commences when Type B spermatogonia lose their contact with the basement membrane to form preleptotene primary spermatocytes. The last mitotic division of the spermatogonia results in the production of the spermatocytes. The spermatocytes push into the adluminal intratubular compartment and enter a lengthy meiotic prophase followed by 2 further rounds of meiotic divisions giving rise to haploid spermatids. This is followed by the transformation of round spermatids into spermatozoa, a process referred to as spermiogenesis. No cell division occurs but the round cell elongates and transforms into a spermatozoa with the capacity for motility. The basic steps in this process are consistent between all species and consists of (a) the formation of the acrosome (b) reorganisation and condensation of nuclear DNA (c) the development of the flagellum (d) the reorganisation of the cytoplasm and cell organelles and (e) the release of the elongate spermatozoa from the Sertoli cell into the tubule lumen (Clermont, 1972; De Rooij et al., 1989; Sharpe, 1994).

1.4. Sertoli cells

The Sertoli cell plays a central role in the development of the testis and in the regulation of spermatogenesis (Sharpe et al., 2003). The Sertoli cells are the first cells to differentiate in the fetal gonad and they are responsible for the organisation of the gonocytes into the seminiferous cords and they prevent the gonocytes from entering into meiosis (Mackay, 2000). Following this, the Sertoli cells continue to proliferate until the initiation of the first wave of spermatogenesis when the Sertoli cells undergo a final differentiation (Griswold, 1998). Sertoli cells provide structural support for the seminiferous epithelium (de Kretser and Kerr, 1988) as well as paracrine factors such as growth factors and other nutrients and provide a unique environment by the formation of junctional barriers (Griswold, 1998).

The regulation of spermatogenesis is mediated via the effects of follicle stimulating hormone (FSH) and testosterone on the Sertoli cells (Figure 1.4). For example, FSH stimulates the Sertoli cells to increase protein synthesis (Griswold, 1998). A key role of FSH during early life is in regulating Sertoli cell number. Experimental studies

have shown that elevated FSH levels can increase Sertoli cell number and thereby increase the spermatogenic output of the testis. (Orth, 1984; Orth et al., 1988).

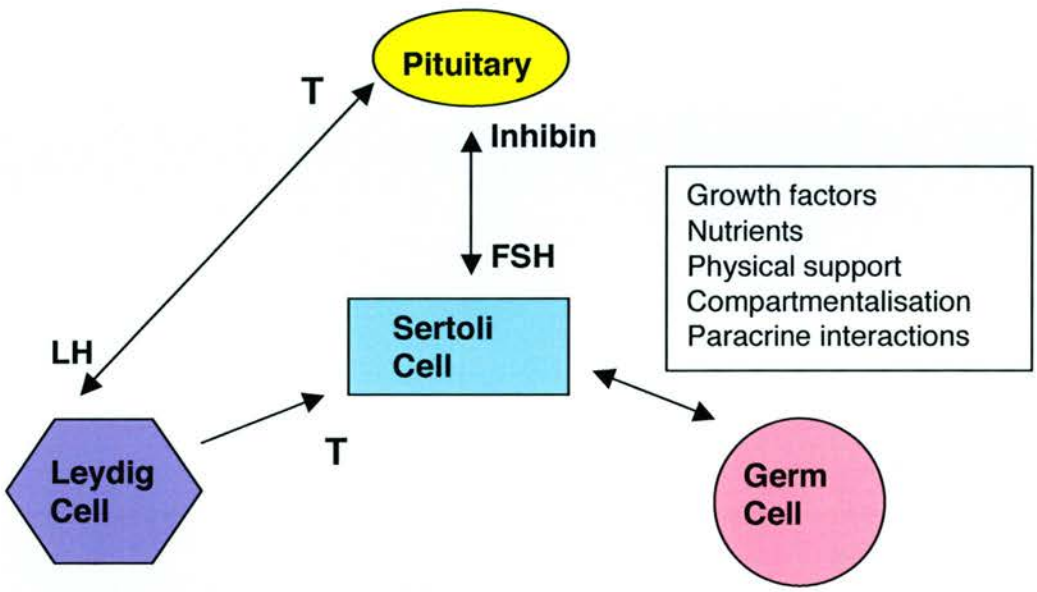


Figure 1.4 The role of the Sertoli cell in Spermatogenesis. Gonadotrophins LH and FSH produced by the pituitary act on the Leydig cells and the Sertoli cells respectively. FSH action on the Sertoli cell leads to the production of testosterone. The many roles of the Sertoli cell in the testis are shown. Adapted from Griswold et al. (2001).

Sertoli cells are capable of supporting only a fixed number of germ cells and therefore the number of Sertoli cells in the testis determines testicular size and the amount of spermatozoa produced (Russell and Peterson, 1984; Sharpe, 1994). There have been no reports of testes that contain germ cells but no Sertoli cells and the ability of germ cells to survive in culture without co-culture with somatic cells is limited (Griswold, 1995; Griswold, 1998). Increasing Sertoli cell number by prolonging the period of proliferation using thyroid hormone also resulted in testis that were larger than normal (Orth et al., 1988). The number of germ cells that each Sertoli cell supports varies between species (Griswold et al., 2001). In rodents and men, Sertoli cells begin to proliferate during fetal development. After birth, the number of Sertoli cells in the rat testis increases 30-fold, which corresponds to a series of approximately five divisions over this period (Wang et al., 1989). The rate of Sertoli cell proliferation decreases steadily after birth in rats and mice from day 5

to 15. In humans and primates with the exception of the rhesus monkey, Sertoli cell proliferation also occurs in the neonatal period with a further proliferative period at puberty (Sharpe et al., 2003). In the rhesus monkey, there is a slightly difference in that it shows the greatest rate of proliferation in the peripubertal period (Marshall and Plant, 1996). After the proliferative period, the Sertoli cells differentiate resulting in morphological changes with the nucleolus becoming more prominent. At the same time, the formation of specialized tight junctions between Sertoli cells establish the blood-testis barrier (Gondos and Berndston, 1993).

The Sertoli cells have a role in regulating the internal environment of the seminiferous tubule. This is facilitated by specialised inter-Sertoli cell junctions which are formed at the sites where processes of Sertoli cell cytoplasm from adjacent cells meet (Dym and Fawcett, 1970). As a result of these junctions, intercellular transport between the Sertoli cell and spermatogonia is possible. The cell junctions divide the seminiferous epithelium into a basal compartment which contains the bases of the Sertoli cells and spermatogonia and an adluminal compartment containing the central regions of the Sertoli cell and the other germ cell types (Figure 1.5). As Type B spermatogonia migrate from the basement membrane of the tubule into the adluminal compartment, the tight junctions open up to allow cellular migration to take place and reform beneath the Type B spermatogonia which have now left the basement membrane to form preleptotene spermatocytes (Lui et al., 2003).

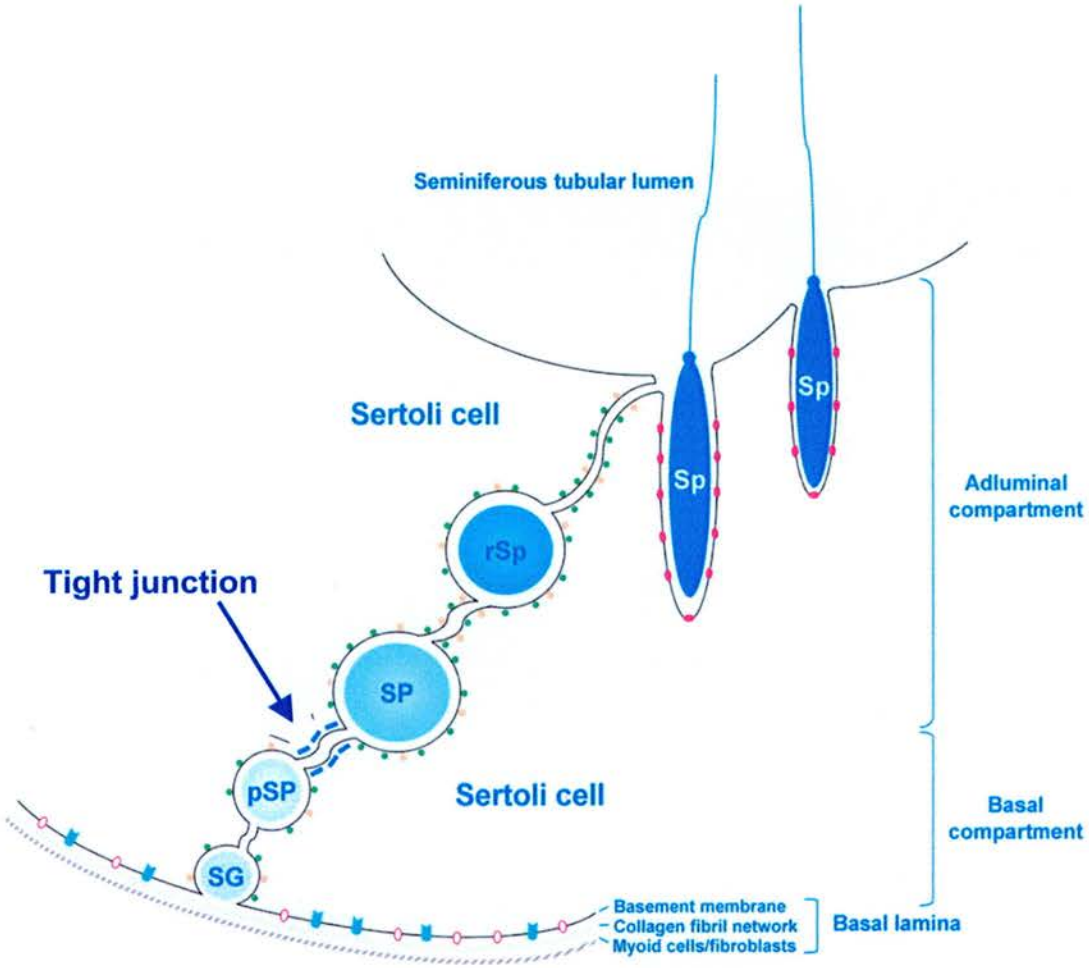


Figure 1.5 Illustration of the two compartments of the seminiferous tubule and the tight junctions between the Sertoli cells in the basal compartments of the testis. Adapted from Lui et al (2003).

Sertoli cells produce more than 100 different proteins and many of these have been identified and characterised (Jegou 1992). For example, Sertoli cells synthesise and secrete inhibins and activins (Roberts et al., 1989); inhibin acts as a negative regulator of FSH secretion by the pituitary and in most species, there is a relationship between the number of Sertoli cells and inhibin concentration (de Kretser et al., 2001).

Androgen receptor (AR) expression is detected in mature Sertoli cells. The AR protein is expressed in a stage specific manner in rats and humans (Bremner et al.,

1994; Suarez-Quian et al., 1999). The expression of AR is switched on prior to final maturation of the Sertoli cell in many species suggesting it may play a role in Sertoli cell maturation (Sharpe et al., 2003). Anti-mullerian hormone (AMH) is among the first genes to be switched on in Sertoli cells (Mackay, 2000). AMH expression in the Sertoli cells continues until puberty, at the time of Sertoli cell maturation when it is down-regulated (Rajpert-De Meyts et al., 1999). In mice, GATA-1 expression is also seen in a stage dependant manner in mature cells (Yomogida et al., 1994). GATA-1 and AMH expression are inversely related and some reports suggest that GATA-1 is responsible for the down-regulation of AMH in Sertoli cells (Beau et al., 2000).

Differentiation of Sertoli cells is also marked by the onset of expression of novel gene products such as transferrin and androgen-binding protein (ABP). ABP acts as a paracrine factor during spermatogenesis and it is known to be involved in testosterone binding and transport (Ritzen et al., 1982). Mature Sertoli cells also secrete sulphated glycoprotein 1 and 2 (SGP-1 and SGP-2) (Collard and Griswold, 1987; Collard et al., 1988).

In most species investigated including rodents and primates, c-kit receptors are expressed on the germ cells. The ligand for the c-kit receptor, known as Steel factor, kit ligand or stem cell factor (SCF) is synthesised by the Sertoli cell. Two forms of SCF have been identified, a soluble and membrane bound form and these are shown in Figure 1.6. In the mouse testis at birth, the membrane bound SCF is the most abundant form found but as development progresses, the soluble form becomes more prevalent (Manova et al., 1993). The c-kit-SCF relationship is important in the regulation of spermatogonial stem cell proliferation and differentiation (de Rooij, 2001; Loveland and Schlatt, 1997) and is discussed further in Chapter 4.

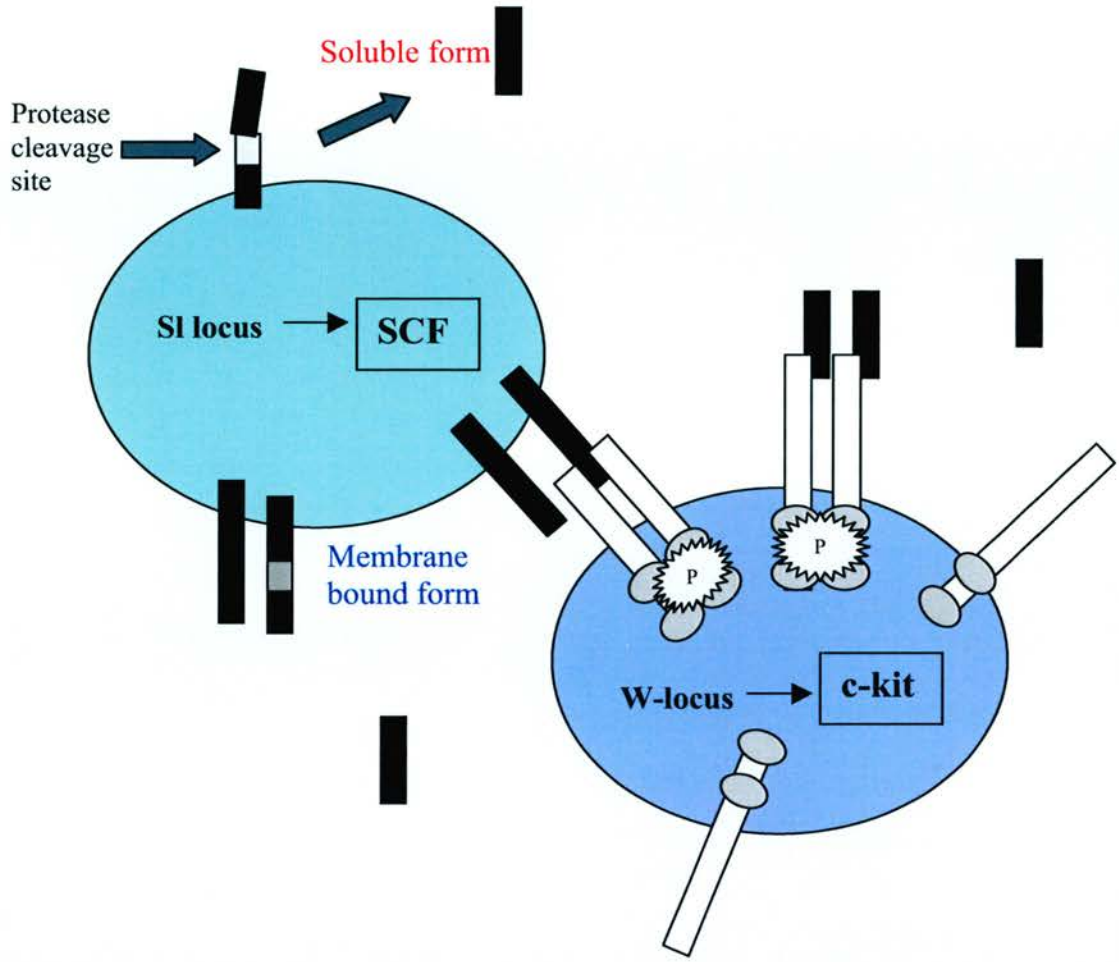


Figure 1.6 SCF is synthesised in the Sertoli cells as one of two isoforms, one form contains a site that can be cleaved with proteases to release a soluble SCF protein while the other form remains membrane bound. SCF (black bars) interacts with c-kit receptor protein (white bars) and this results in phosphorylation (P) of c-kit to effect signal transduction. (Adapted from Loveland and Schlatt 1997).

Pem is expressed in adult mouse Sertoli cells, its gene expression has been shown to be androgen and gonadotrophin dependent (Lindsey and Wilkinson, 1996; Sutton et al., 1998). Pem is a transcription factor that mediates androgen regulated events in spermatogenesis and is dramatically induced at the initiation of meiosis during the first wave of spermatogenesis (Wayne et al., 2002). Pem knockout mice show normal development and spermatogenesis suggesting that the role of Pem is not critical in normal spermatogenesis (Pitman et al., 1998). However studies on Pem overexpression mice in Wayne et al. (2002) have suggested that Pem regulates the

expression of Sertoli cell proteins that control DNA modification events in germ cells. The results showed that constitutively expressing Pem in Sertoli cells in all stages of the seminiferous epithelial cycle, results in a dramatic increase in the number of DNA-strand breaks in specific germ cell populations (Wayne et al., 2002).

1.5. Germ cell – Sertoli cell interactions

The fact that the testis has a dual function with the production of hormones and the production of spermatogenesis suggests a large amount of cell-to-cell communication is occurring in the testis (Schlatt et al., 1997). Germ cells and Sertoli cells begin to interact with each other very early during testicular development and this essential relationship continues throughout spermatogenesis to the production of spermatozoa (Griswold, 1995; Griswold, 1998; Saunders, 2003). One of the most important roles of Sertoli cells is the regulation of the intratubular and intercellular environment. Paracrine and autocrine factors from Sertoli and germ cells are important in the functioning of both cell types. The primary endocrine regulation of spermatogenesis is via FSH and testosterone acting upon the Sertoli cells (Griswold, 1995) and this is discussed later in this chapter (section 1.7.1.1). The relationship between the c-kit receptor found on the germ cells and its Sertoli cell expressed ligand SCF, which is discussed further in Chapter 4 and in section 1.4, is also very important in the regulation of spermatogenesis (Loveland and Schlatt, 1997; Manova et al., 1993).

It has recently been shown that germ cells from mice deficient in the enzyme encoding alpha-mannosidase 11x failed to adhere to Sertoli cells and underwent premature release into the epididymis implicating both a role for carbohydrates in the interactions between germ cells and Sertoli cells but also the importance of the role that the Sertoli cell plays in the physical support of the germ cell (Akama et al., 2002).

1.6. Germ cells *in vitro* and *in vivo*

In the adult mouse testis there are around 35,000 stem cells which accounts for 0.03% of all germ cells (Tegelenbosch and de Rooij, 1993). Very little is known

about these cells due mainly to a lack of specific markers. Various methods have been used to purify or enrich the stem cell population, for example, cells have been isolated from vitamin A deficient rats (van Pelt et al., 1996). In these rats, spermatogenesis is arrested at the differentiation step of A_{al} into A_1 spermatogonia so only A_s, A_{pr} and A_{al} spermatogonia are present within the testis. Brinster and Shinohara showed that the stem cell population express the cell surface markers α-6 integrin and β-1 integrin (Shinohara et al., 2000b) and this information has been important in studies which have attempted to enrich the stem cell population, which are described in section 1.6.2.2.

1.6.1. Germ cell cultures

Mammalian spermatogenesis has yet to be demonstrated in full in a cell culture system. Although germ cells are able to survive in culture for long periods of time, attempts to expand and proliferate germ cells in culture have proved on the whole unsuccessful. Initial studies by Brinster and colleagues showed long term survival of stem cells when they were cultured in the presence of serum and on a STO feeder layer (Nagano et al., 1998). No proliferation of the stem cell population was observed and the survival rate of the cells fell dramatically with only 10-20% of the stem cells remaining in culture after a week (Nagano et al., 1998). However, after germ cell transplantation (section 1.6.2), the cells were able to re-populate a sterile recipient mouse showing that long term culture of stem cells did not result in differentiation of the cells or loss of stem cell characteristics (Nagano et al., 1998). Long term survival of the stem cells and more importantly, proliferation of the stem cell population has also been shown when the cells are co-cultured with Sertoli cells (van der Wee et al., 2001)

Attempts to immortalise stem cells using telomerase and SV40 T antigen have also been made with proliferation of the cells being reported (Feng et al., 2002; van Pelt et al., 2002). It is unclear however whether these cells retain true stem cell potential as to date, no transplantation assays have been performed on these cells. A study by Kanatsu-Shinohara and colleagues (2003) showed that long-term culture of cells with various growth factors could proliferate and restore fertility in mice transplanted with

these cells. They found that a combination of growth factors (GDNF, bFGF, EGF and LIF) could induce the proliferation of stem cells in vitro. After long term culture, they found that the cells still expressed α-6 and β-1 integrin (Kanatsu-Shinohara et al., 2003), known to be markers of spermatogonial stem cells (Shinohara et al., 1999), and were negative for c-kit (Schrans-Stassen et al., 1999), which is a marker of differentiated spermatogonia in the mouse. The cells were also negative for SSEA-1, which is a PCG marker (Resnick et al., 1992), indicating that the cells were undifferentiated spermatogonial stem cells.

1.6.1.1. Germ cell lines

The establishment of germ cell lines could be very useful in the study of germ cell function during the process of spermatogenesis. Attempts have been made to immortalise germ cell cultures with varying amounts of success. The GC-1 cell line (Hofmann et al., 1992) was made by immortalising mouse germ cells using the SV40 large-T antigen. These cells corresponded to a stage between B type spermatogonia and primary spermatocytes and were found to express testicular cytochrome c_t and lactate dehydrogenase-C_4 (LDH-C4), both markers of meiotic and post meiotic cells. These cells failed to differentiate in vitro and this was thought to be due to the presence of the large T antigen. The same group reported two years later, that a second germ cell line, GC-2 could undergo meiosis in vitro. These cells were immortalised using the SV40 large T antigen together with the gene encoding the temperature sensitive p53 mutant. At the non-permissive temperature of 39°C, p53 is inactive. Culture at 37°C or below abolishes the proliferative function of the large T antigen allowing the cells to differentiate. The GC-2 cells had the morphologic and biochemical appearance of round spermatids (Hofmann et al., 1994). At 39°C the markers testicular cytochrome c_t and LDH-C4 are expressed weakly. Dropping the temperature to 37°C or 32°C, resulted in increased expression of these markers indicating that the cells had differentiated and entered meiosis. However, subsequent investigation of these cells (Wolkowicz et al., 1996) found that over time, the cells had become arrested and were no longer capable of differentiation. Although not strictly a differentiating cell line, they still represent a useful tool in studying germ cells.

1.6.1.2. Retroviral immortalisation of germ cells

Martin Dym's group reported the development of a new immortalised germ cell line in 2002 (Feng et al., 2002). In their studies, undifferentiated mouse spermatogonia were retrovirally transfected with mTERT. The male germline stem cell expresses high levels of telomerase activity, but this expression decreases during germ cell differentiation (Ravindranath et al., 1997). It has been shown that telomerase deficiency in mice leads to a depletion of male germ cells (Lee et al., 1998) and that ectopic expression of the telomerase catalytic component, TERT, in certain cell types can extend their life-span and immortalize them (Hahn et al., 1999; Morales et al., 1999). From this information, it was suggested that spermatogonia may be maintained in an undifferentiated status by overexpression of TERT in vitro (Feng et al., 2002). After 2 months in culture, the cells were still capable of proliferation while still exhibiting type A spermatogonia morphology. The cells expressed Oct-4, Dazl and c-kit, all markers of spermatogonia. Culture of the cells in media containing mSCF induced the cells to enter meiosis, thereafter cells expressed LDH-C4, and the spermatid markers SP-10 and protamine-2 (Feng et al., 2002).

1.6.2. Spermatogonial stem cell transplantation

Undifferentiated spermatogonia remain totipotent and undergo self-renewal. The method of stem cell transplantation was pioneered in mice by Ralph Brinster and his group in 1994 (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994) and provided a functional assay of whether a putative stem cell population are true "stem" cells.

The method of germ cell transplantation involves the isolation of testicular cells from a fertile mouse and the subsequent transplantation of the cells back into the lumen of the testis of an infertile recipient mouse with a similar genetic background. The transplanted donor stem cells then repopulate the seminiferous epithelium of the recipient testis reinitiating spermatogenesis (Brinster and Zimmermann, 1994). The initial experiments carried out by Brinster transplanted cells from young mice between 4 and 12 days old as these contain a higher proportion of spermatogonia compared with adults. Two different strains of mice were used as recipients, the first,

W mutant mice, were selected as these mice are sterile because of the lack of differentiated germ cells and spermatogonial stem cells due to a mutation in the c-kit locus (Besmer et al., 1993; Brinster and Zimmermann, 1994). The second group of mice were made infertile following treatment with busulphan. Treatment with busulphan, which is a chemotoxic agent results in destruction of the spermatogenic stem cells (Bucci and Meistrich, 1987). Four weeks after busulphan treatment, no signs of spermatogenesis are present and testis size is significantly reduced to about 10-15% of a size of normal (Brinster and Zimmermann, 1994). Chemical sterilisation of the testis by treatment with busulphan has to be treated with caution, as this method is not always 100% effective in eliminating all the stem cells. To overcome this, donor mice expressing lac-z (Rosa) (Zambrowicz et al., 1997) or GFP within their germ cells (Okabe et al., 1997) have been useful in identifying whether the germ cell colonies formed after transplantation are donor derived or from re-initiation of endogenous spermatogenesis.

In order to perform transplantation, donor cells are isolated by enzymatic digestion and the resulting single cell suspension is injected into the testis via the efferent ducts (Brinster and Zimmermann, 1994). Transplanted cells then move from the lumen of the tubule to the basal compartment of the testis where a stem cell niche is believed to exist, the stem cell niche is described further in section 1.6.2.1 (Brinster, 2002). Germ cell movement is thought to be mediated by the Sertoli cells, which transport the transplanted cells into their usual position in the tubule. This involves movement of the stem cells through the blood testis barrier in the opposite direction from normal. The blood testis barrier consists largely of inter-Sertoli cell tight junctions and is shown in Figure 1.5. As this process must involve breaking down and re-forming tight junctions between Sertoli cells it seems likely that the Sertoli cells are able to recognise spermatogonial stem cells (Griswold, 1998).

After the initial success of the transplantation experiments, it was found that spermatozoa from donor-derived colonies were capable of fertilising oocytes and producing progeny which carried the donor haplotype (Brinster, 2002; Brinster and Avarbock, 1994). Additional studies reported that germ cell suspensions could be

frozen and successfully transplanted at a later date (Avarbock et al., 1996). This has given hope that this technique could be used as a treatment for infertility resulting from treatments such as radio- or chemotherapy, which destroy endogenous stem cells rendering patients infertile. For example, testicular germ cells could be recovered from pre-pubescent boys, frozen until required and then thawed and transplanted at a later day. This proposal is not without controversy, the major concern being that cancerous cells could be reintroduced.

Since the first report of a successful spermatogonial cell transplant, many modifications to the procedure have been carried out, both improving the efficiency of the procedure and also establishing the technique in other species. Enrichment of the stem cell population to improve colonisation rates has been very successful and methods used in achieving this are discussed in section 1.6.2.2. To overcome the problems associated with the use of busulphan, many groups have moved towards using mouse models of infertility. Many mouse models showing arrested spermatogenesis have been described (Cooke and Saunders, 2002) and several of these have been successfully used as recipients for transplantation including the Steel and Dazl mouse mutants (Ogawa et al., 2000b; Rilianawati et al., 2003). Transplants have also been undertaken in other species including rats, hamsters and primates (Jiang and Short, 1995; Schlatt, 2002; Schlatt et al., 1999). Modifications to the transplant procedure had to be undertaken in primates with the cells being injected into the rete testis guided by ultrasound (Schlatt et al., 2002; Schlatt et al., 1999).

As spermatogonial stem cells appear to be similar in many species (Russell et al., 1990), the ability of spermatogonia to colonise seminiferous tubules of another species has been investigated with colonisation of transplanted donor cells being observed in rat to mouse, mouse to rat, hamster to mouse, primate to mouse, and rabbit to mouse transplants (Clouthier et al., 1996; Dobrinski et al., 1999; Nagano et al., 2001b; Ogawa et al., 1999; Schlatt et al., 1999; Zhang et al., 2003). The most successful intra-specific transplants that have been reported have been from the mouse to the rat. Cells from transgenic mice were transplanted to the testes of immunodeficient rats, resulting in restoration of spermatogenesis with the production

of spermatozoa with normal mouse morphology being seen in the epididymis of the rat (Clouthier et al., 1996; Zhang et al., 2003). This was an important development as it showed rat germ cells retained their 52-day cycle after transfer to the mouse testis and it confirmed reports claiming it is the germ cells and not the Sertoli cell environment that controls the length of the spermatogenic cycle (Franca et al., 1998; Russell and Brinster, 1996). Other intraspecific transplants have been less successful. Ogawa et al. (1999) reported that hamster spermatogenesis could be produced in an immunodeficient mouse recipient. However, this resulted in the production of elongated spermatids with abnormal head shapes (Ogawa et al., 1999). No spermatozoa were produced from xenogeneic transplantations from rabbit to mouse and from dog to mouse The transplanted cells, moved to the basal compartment but did produce mature germ cells. (Dobrinski et al., 1999). It has been hypothesized that the success of transplants is correlated to the degree of evolutionary relatedness of the species (Johnston et al., 2000).

Although intraspecies transplantation represents a significant step forward in the application of the methods to human patients, success has so far been limited. One of the first attempts to transplant human germ cells into testes of immunodeficient mice showed that no human germ cells survived in mouse testes, possibly due to immunological rejection or incompatibility between human germ cells and mouse somatic cells (Reis et al., 2000). A subsequent study by Nagano (2002) demonstrated colonisation of human spermatogonial stem cells within the testes of immunodeficient mice for at least 6 months after transplantation but did not differentiate beyond undifferentiated spermatogonia in mouse testes (Nagano et al., 2002).

1.6.2.1. Stem cell niche

In the haematopoietic system, stem cell microenvironments termed "niches" were described (Schofield, 1978). A niche is considered to be a subset of cells and extracellular substrates that can indefinitely house stem cells and control their self-renewal and progeny production. Demonstration that a niche is present requires that after stem cells removal, transplanted cells will begin to repopulate the tissue. It is

said that a niche should persist in the absence or removal of stem cells and support the stem cell activity of competent exogenous cells (Spradling et al., 2001). The existence of such a niche in the testis was demonstrated when the process of germ cell transplantation was developed (Shinohara et al., 2001). Once cells are transplanted, they migrate down into the tubule and pass through the Sertoli cell tight junctions and colonise sites on the basement membrane of the tubule. It is thought that these cells occupy empty niches that lie in abundance along the depleted basal layer (Spradling et al., 2001). Each colony seems to be established from an individual spermatogonial stem cell. It has been proposed that the number of colonies formed was representative of the number of niches that exist along the basement membrane of the seminiferous tubule (Spradling et al., 2001).

Niche number and quality seem to change during development (Spradling et al., 2001). Evidence for this comes from studies involving the W mutant mice, which lack c-kit function and exhibit abnormal spermatogenesis (Shinohara et al., 2001). When these mice are used as recipients for germ cell transplantation, their testes support more colonies of donor-derived germ cells when they are prepubertal (between days 5-15) compared with recipients used in adulthood, regardless of the age of the donor cells. This suggests that niches may be more abundant or easier to access in younger animals (Ohta et al., 2000; Shinohara et al., 2001). Integrins (α_6 / β_1) are enriched in the basal component of the seminiferous tubule (Shinohara et al., 1999) and it has been proposed that these molecules may aid attachment of the spermatogonial stem cells to laminin in the basement membrane and thereby participate in the niche.

1.6.2.2. Enrichment of the stem cell population

The stem cell population in the adult mouse testis is small, representing only 1 in 4000 of the total germ cell complement (Tegelenbosch and de Rooij, 1993). Several approaches have been used both to identify spermatogonial stem cell markers and in the isolation of the stem cell population (Kubota et al., 2003). For example, cells expressing surface markers have been isolated using immunomagnetic beads and transplanted into recipient testes. It was found that selection of germ cells that

expressed α-6 integrin and β1 integrin on their surface prior to transplantation increased the number of colonies formed following transplantation by 166 fold compared to an unselected population (Shinohara et al., 1999).

Enrichment of the stem cell population can also be achieved by isolating cells from mutant mice such as the steel mutant, which are infertile as the testes of homozygous mutants are virtually devoid of germ cells (Shinohara et al., 2000a) or vitamin A deficient mice (VAD) which results in a loss of mature germ cells in the testis, resulting in infertility (Griswold et al., 1989; van Pelt and de Rooij, 1990). Cells derived from steel mice have been used as successful donors for transplantation and spermatogenesis could be restarted in the VAD mice after treatment with vitamin A. This suggests that the stem cell population remains in these animals and the use of these mice for isolation of germ cells would result in a population that is enriched for the stem cell population. Other ways to enrich the stem cell population have been achieved by making mice cryptorchid or exposing the testis to high temperatures (McLean et al., 2002; Shinohara et al., 2000a). Cryptorchidism, either naturally occurring or experimentally achieved by retaining the testis in the body cavity of the mouse, results in the loss of mature germ cells (Nishimune and Aizawa, 1978; Nishimune et al., 1986). Similarly, testes of mice who underwent scrotal heating at 43°C for 15 minutes lack meiotic and post meiotic germ cells (Chowdhury and Steinberger, 1964). Transplantation of donor cells derived from the testes of hyperthermic mice resulted in a 5.3 fold increase in the number of colonies and the area of colonisation was increased 19 fold (McLean et al., 2002). Transplantation of cells isolated from cryptorchid testes resulted in a 25 fold increase in the number of colonies and a 50 fold increase in the area of spermatogenesis compared with control transplantations carried out with cells derived from normal animals (Shinohara et al., 2000a). In contrast, the use of germ cell mixtures from VAD mice only resulted in a 2.5 fold increase in the number of colonies (McLean et al., 2002) and the use of the steel mutant mouse was reported as giving a modest increase in donor derived colonies of spermatogenesis compared to control (Shinohara et al., 2000a). The results from these experiments tell us that the proportion of germ cells representing

the stem cell population can be successfully enriched by the use of mouse models that lack more mature germ cells.

1.6.3. Manipulation of stem cells

Genetic manipulation of the stem cells prior to transplantation could provide a quicker and easier way of generating transgenic mice than traditional methods and would allow transgenesis through the male germ line instead of the female. One of the first successful reports using this approach used testicular cells that were cultured with a retroviral vector before being used for transplantation. The viral vectors were able to integrate into the chromosomal DNA of the stem cells and Nagano and co-workers reported that at least 1 in 300 stem cells were infected with the retrovirus and virus expression remained detectable for more than 6 months (Nagano et al., 2000). Following on from this study, retrovirally infected cells were transplanted into infertile recipient mice resulting in stable integration of the transgene in 2-20% of the stem cells and the transgene was transmitted through the germline to the offspring (Nagano et al., 2001a). Stem cells from the rat are also receptive to retroviral infection with 0.5% of cultured then infected stem cells producing colonies of spermatogenesis after germ cell transplantation (Orwig et al., 2002).

1.7. Steroidogenesis

In the male, the testis is a major site of synthesis of steroid hormones, including oestrogens and androgens. Steroid biosynthesis is summarised in Figure 1.7. The pathway from cholesterol to androstenedione is the same in both the testis and the adrenal gland and uses the same enzymes. Testicular testosterone is produced in the Leydig cells. The enzyme 3ß-hydroxysteroid dehydrogenase (3ßHSD) is responsible for the conversion of pregnenolone to progesterone, 17-hydroxypregnenolone (17OH-pregnenolone) to 17-hydroxyprogesterone (17OH-progesterone), dehydroepiandrosterone (DHEA) to androstenedione, and androstenediol to testosterone. P450c17 is also necessary in the proximal stages of testosterone synthesis. P450c17 catalyzes the 17-hydroxylation of pregnenolone to 17OH-pregnenolone and progesterone to 17OH-progesterone. The conversion of androstenedione to testosterone in the testis is catalysed by 17ßHSD type 3

(17ßHSDIII). The testosterone secreted from the Leydig cells can enter the Sertoli cells where it can be further converted to dihydrotestosterone (DHT) by 5α reductase (Holmes et al., 2004; Miller, 1988).

Although at first widely believed to be female specific hormones, there is now evidence that oestrogens are synthesised in the male reproductive tract by the Sertoli cells, the Leydig cells and the germ cells (Hess, 2003; Hess et al., 2001). In the male, oestrogen is synthesised from testosterone by the enzyme cytochrome P450 aromatase or oestrone by 17β-hydroxysteroid dehydrogenase (17β-HSD) (Figure 1.7) in the brain and in the testis (de Ronde et al., 2003). It was the discovery of aromatase in the testis that led to the suggestion that oestrogens might play a role in male reproductive function (Hess, 2003). In mammals, cytochrome P450 aromatase, a product of the CYP19 gene has been localised to the Leydig cells in many species including man, mouse, rat, ram and pig (Carreau et al., 2003; Conley and Hinshelwood, 2001; Inkster et al., 1995; Kurosumi et al., 1985; Nitta et al., 1993). In the mouse, aromatase expression is also seen in the Sertoli cells and in the germ cells (Nitta et al., 1993) and aromatase is present in the germ cells of both humans and primates (Lambard et al., 2004; Turner et al., 2002).

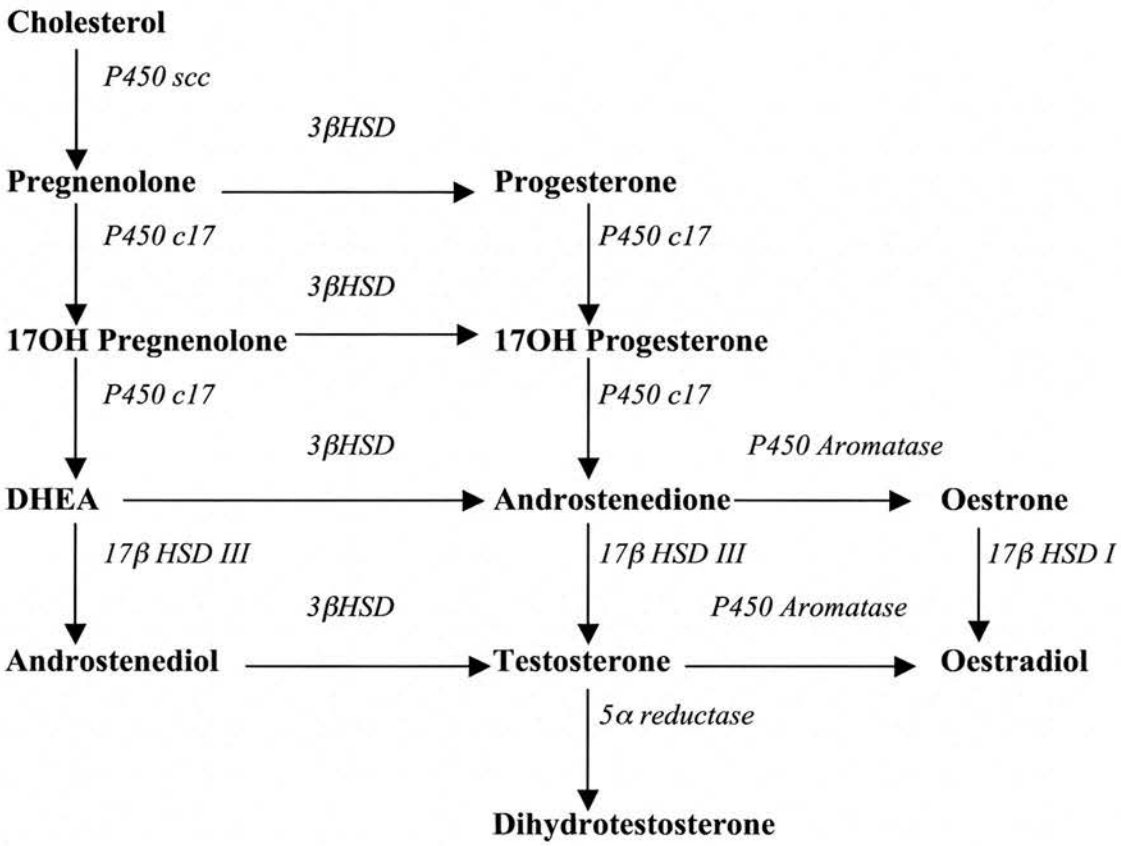


Figure 1.7 Steroid synthesis from cholesterol. Adapted from Holmes et al., 2004.

1.7.1. Hormonal regulation of male fertility

The hypothalamus, pituitary gland and gonads regulate the development of reproductive function in mammals. Gonadotrophins and steroid hormones along with other intratesticular modulators are responsible for the initiation and maintenance of spermatogenesis in the mammalian testis (Carreau et al., 1999; Sharpe, 1993). Figure 1.8 summarises the interactions of the gonadotrophins and the steroid hormones in the testis. The secretion of steroid hormones, especially testosterone is necessary for the expression of secondary sexual characteristics and the production of spermatozoa. Testosterone is essential for spermatogenesis demonstrating that paracrine signals between testicular cell types is essential and suggests a role for AR in spermatogenesis (Sharpe et al., 1988). The secretion of steroid hormones is

dependant on stimulation by the gonadotrophins, FSH and LH (McLachlan et al., 1996). The review by McLachlan (1996) gives examples of studies that show spermatogenesis does not proceed normally if the testis is not stimulated by these hormones. The *hpg* mouse is an example of this, a naturally occurring deletion in the GnRH gene results in a model of hereditary hypogonadism (*hpg*), both sexes of these mice are infertile due to immature gonads. The mice completely lack gonadotrophin releasing hormone (GnRH), resulting in severely reduced levels of FSH and LH within the pituitary (Cattanach et al., 1977). The consequence of this loss of gonadotrophin secretion is a reduction in testosterone biosynthesis by the Leydig cells.

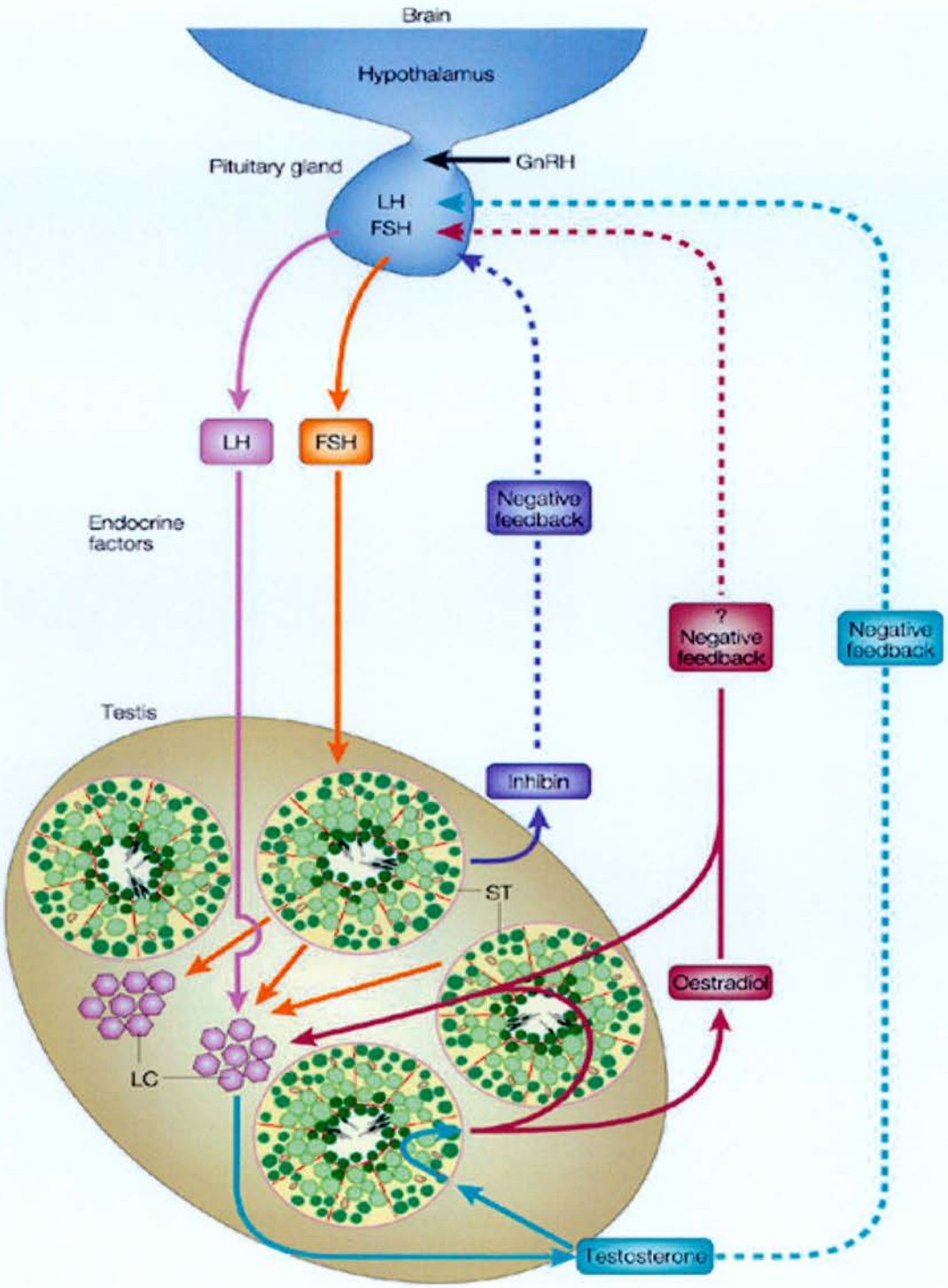


Figure 1.8 Hormonal control of the male reproductive tract. Taken from Cooke and Saunders (2002).

1.7.1.1. FSH and LH

LH and FSH both act through different specific G-protein coupled transmembrane receptors (Simoni et al., 1997a; Simoni et al., 1997b). The hypothalamic secretion of GnRH stimulates the anterior pituitary to release FSH and LH. LH travels through the bloodstream and binds to the LH receptor present on Leydig cells where it stimulates biosynthesis of testosterone. LH also stimulates the synthesis of steroidogenic acute regulatory protein (StAR), which is involved in the acceleration of the transfer of cholesterol from the outer to the inner mitochondrial membrane prior to the first step in steroid biosynthesis. Inhibin B is a major hormonal product of the testis (Illingworth et al., 1996). Inhibin B is produced in the Sertoli cells under the stimulatory influence of FSH and acts to inhibit FSH secretion from the pituitary (Anawalt et al., 1996).

Some studies have suggested that FSH is the primary endocrine hormone involved in Sertoli cell regulation. As early as 1937, FSH was demonstrated to be able to maintain spermatogenesis in hypophysectomised rats (Greep and Fevold, 1937). In man, Matsumoto et al. (1986) reported that FSH treatment was able to restore sperm counts in men who had suppressed spermatogenesis caused by a lack of gonadotrophin secretion (Matsumoto et al., 1986). During development, FSH stimulates seminiferous tubule growth and is a major determinant of adult testicular size (Amory and Bremner, 2003) because FSH influences Sertoli cell number and the induction and maintenance of normal spermatogenesis (Simoni et al., 1999). In the human, there is evidence to suggest that FSH may also play a role in the regulation of the development of the adult Leydig cell population (Baker et al., 2003a; Sharpe, 1993). Sertoli cells are stimulated by FSH via the FSH receptor and FSH has been shown to regulate the expression of many Sertoli cell products including androgen binding protein, transferrin and aromatase (Griswold, 1998; Griswold et al., 1988; Skinner, 1991). Further evidence that FSH and androgens are both required for efficient Sertoli cell function is provided by studies in knockout animals and the effects of the lack of FSH and androgens in these models are discussed in section 1.9.

1.7.1.2. Androgens

From the Leydig cells, testosterone is released into the bloodstream and also travels to the Sertoli cells where it can be converted to its more active metabolite DHT (Figure 1.7). Although the affinity of DHT for the androgen receptor is said to be twice that of testosterone, it is believed that testosterone is the predominant ligand for the androgen receptor (Wright and Frankel, 1979). The relative abundance of testosterone in the testis is around five times greater than DHT (Wright and Frankel, 1979).

It has been proposed that FSH and testosterone work together in the regulation of spermatogenesis and may share a common post receptor pathway (Russell and Griswold, 1993). Huang et al. proposed the idea that FSH played a role in transporting and localising testosterone within the Sertoli cells in association with ABP (Huang et al., 1991). The importance of testosterone for normal spermatogenesis has been endorsed by studies showing its withdrawal results in the degeneration of spermatogenic cells (McLachlan et al., 1996). Following treatment with ethane dimethane sulphonate (EDS) which is a Leydig cell cytotoxin, pyknotic nuclei and cytoplasmic vacuoles, both signs of cellular degradation were observed within cells, particularly in the spermatocytes and spermatids (Kerr et al., 1993).

The studies discussed above involving testosterone were performed on rodent models and it was hoped that by elucidating the role of testosterone, advances would be made in the human eventually leading to the use of androgens, especially testosterone as a male contraceptive. The basis behind this is that high levels of testosterone decreases LH and FSH secretion leading to a reduction in testicular testosterone by negative feedback and the cessation of spermatogenesis which in turn results in a decrease in fertility (Amory and Bremner, 2003). Clinical trials using exogenously administered testosterone to suppress FSH and LH levels as a hormonal male contraceptive have induced spermatogenic suppression in a majority of men. A recent report in which subjects were given weekly injections of the testosterone ester testosterone enanthate, azoospermia was achieved in only two-thirds of Caucasian

men (Amory and Bremner, 2003; Kamischke and Nieschlag, 2004). However oligozoospermia is achieved in a majority of cases and the failure rate in these cases is low (3.5%). One of the main advantages to hormonal contraception in the male is that it is reversible and sperm counts are found to return to normal after cessation of testosterone treatment. Progress is also being made using testosterone in combination with different progestins or gonadotrophin-releasing-hormone antagonists. Of these, testosterone administered in combination with either depot medroxyprogesterone acetate, norethisterone enanthate, desogestrel or etonogestrel have shown promising efficacy (Kamischke and Nieschlag, 2004).

1.7.1.3. Oestrogens

The importance of oestrogens in the male was first demonstrated when malformations in the male reproductive tract were observed following exposure to potent oestrogens (Arai et al., 1983). This was followed by the discovery of the aromatase complex in the testis and problems with fertility in mice with targeted deletions in the ERα and aromatase P450 (cyp19) genes (Lubahn et al., 1993; Robertson et al., 1999). The production rate of oestradiol in the adult human male has been estimated to be 35-45μg per day and 15-20% of this is produced by the testes (Baird et al., 1969). In the testis, both the immature Sertoli cells and the Leydig cells synthesise oestrogens and it has been shown that the germ cells of many species including rodents, humans and primates are also a source of oestrogens (Carreau et al., 1999; Nitta et al., 1993; Turner et al., 2002).

Oestrogens play a major role in many metabolic processes in the male outside the testis including the cardiovascular system and in bone homeostasis. Patients with oestrogen insensitivity or aromatase deficiency show decreased bone mineral density (de Ronde et al., 2003; Morishima et al., 1995; Smith et al., 1994). A report by Carlsen and co-workers in 2000 suggests that male osteoporosis is associated with low peripheral oestradiol levels (Carlsen et al., 2000). In another study in young patients with unexplained osteoporosis but normal oestrogen levels, a reduction in the level of expression of ERα protein in osteoblasts and osteocytes was seen and it was suggested this could explain the bone loss (Braidman et al., 2000). Oestrogen

receptors are also expressed throughout the cardiovascular system (Gray et al., 2001). In addition, both the aromatase knockout mice and human patients with a mutation in the *CYP19* gene show obesity, hypercholesterolaemia and hypertriglyceridaemia (Fisher et al., 1998). These phenotypes add evidence to the suggestion that oestrogens play a role in the cardiovascular system.

1.8. Steroid hormone receptors

The gonads and adrenal glands produce five major groups of steroid hormones, oestrogens, androgens, progestin's, glucocorticoids and mineralocorticoids. In mammals, the actions of oestrogens and androgens are mediated through members of the steroid hormone receptor super family (Parker, 1991) expressed in target cells. Classical steroid hormones receptors are transcription factors that are activated by ligand binding (Parker, 1990), ligand binding promotes receptor dimerisation and high affinity binding to chromatin organised DNA sequences termed hormone response elements (Beato and Klug, 2000; Mangelsdorf et al., 1995). The HRE-receptor complexes are then able to modulate transcription of target genes. Members of the steroid hormone receptor family share significant sequence homology and are characterised by a similar domain structure (Figure 1.10). Briefly, the receptors all contain a DNA binding domain (DBD), which contains two zinc finger domains, which are important in targeting the receptor to the HRE. The ligand binding domain (LBD) is situated at the c-terminal end of the receptor and it is this region that binds specific hormonal and non-hormonal ligands (Olefsky, 2001). Ligand binding also activates two different activation functions, the first, termed activation function 1 (AF-1) is located in the A/B region in the N terminal domain and the second, activation function 2 (AF-2), which is a ligand-inducible activation function close to the LBD. The two AF's act to connect the receptor to transcriptional apparatus via transcription factors, co-activators and co-repressors (Beato and Klug, 2000). Oestrogen receptors were first characterised by Jensen and DeSombre in the 1970's (Jensen and DeSombre, 1973) and this led to the cloning of the receptors in the 1980's. The first oestrogen receptor, now known as ERα was discovered in 1958 and cloned in 1986 from a human breast cancer cell line (Green et al., 1986). For several years it was believed to be the only form that existed. However, in 1996, a second

form was cloned by degenerative PCR from rat prostate and named ERβ (Kuiper et al., 1996).

The androgen receptor is also expressed throughout the male reproductive tract. AR functions as a ligand-dependent transcription factor, regulating expression of an array of target genes that are important in male development and fertility (Collins et al., 2003). Regulation of androgen production via the hypothalamic-pituitary-gonadal axis is necessary for development of the male phenotype, as well as for initiation and maintenance of spermatogenesis. AR activity is regulated by the steroid ligand testosterone and its derivative dihydrotestosterone (DHT), the binding of which initiates nuclear translocation and the transcriptional regulatory function of AR (Lindzey et al., 1994), and this can mediate a wide range of physiological responses and developmental processes. The ligand bound AR can then form homodimers and bind specific DNA elements referred to as androgen response elements (ARE) in target gene promoters. After ligand binding, the AR can also interact with various factors (e.g. co-activators or co-repressors) to modulate transcription of androgen target genes via specific DNA sequences (Lee and Chang, 2003). AR plays an important role in the feedback regulation of Testosterone levels. This regulation occurs through autocrine feedback on the Leydig cells, via effects on GnRH production, and through inhibition of LH secretion by the pituitary (Amory and Bremner, 2001). The androgen receptor was cloned in 1988. It was localised to the X-chromosome and has a similar domain structure to other members of the nuclear receptor superfamily, shown in Figure 1.10 (Lubahn et al., 1988).

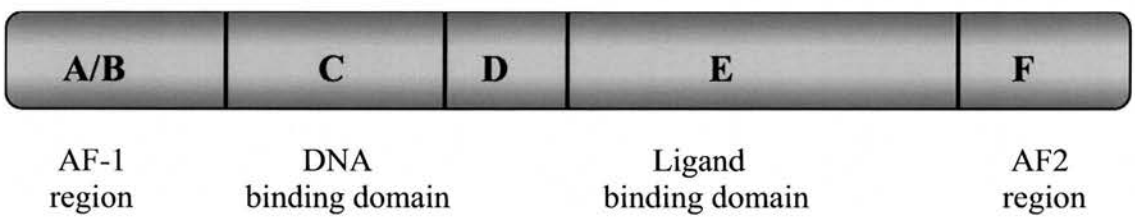


Figure 1.10 Domain structure of a steroid hormone nuclear receptor.

1.8.1. Expression of steroid hormone receptors within the testis

Cellular localisation of both the androgen and the oestrogen receptors within the male has led to a better understanding of their involvement in normal physiology (Couse and Korach, 1999). Both ERα and ERβ bind oestradiol and bind an oestrogen response element (ERE), 5'-GGTCAnnnTGACC-3', with high affinity (Klinge, 2001). It has been shown that ERα and ERβ bind ligands with different affinities and are distinct in their transcriptional activities (Katzenellenbogen and Katzenellenbogen, 2000; Kuiper and Gustafsson, 1997; Paech et al., 1997). The difference in the way that the two receptors are activated suggests further that both receptors play different roles in gene regulation (Paech et al., 1997).

1.8.1.1. Oestrogen receptor alpha (ERα)

ERα is expressed in many tissues in the mouse including the ovary, uterus, oviduct and mammary gland in the female and in the testis, prostate and epididymis of the male (Couse and Korach, 1999; Zhou et al., 2002). ERα is also expressed in non-reproductive organs including the heart, liver, kidney and lung (Brandenberger et al., 1998; Couse et al., 1997; Kuiper et al., 1997). In the murine testis, ERα has been localised to the Leydig cells and the peritubular myoid cells. No expression has been detected in Sertoli cells or germ cells (Zhou et al., 2002). High levels of expression have been detected in the efferent ductules (Cooke et al., 1991).

In the rat, ERα is again highly expressed in the efferent ductules. Adult Leydig cells are also immunopositive for ERα, but ERα is not expressed in Sertoli cells, peritubular myoid cells, blood vessels or germ cells at any time (Fisher et al., 1997). In dogs and cats, no expression of ERα has been detected in Sertoli cells or germ cells. Cats also showed no expression in the peritubular cells and the only expression of ERα was in the interstitial cells while dogs showed both interstitial cell and peritubular myoid cells expression of ERα protein (Nie et al., 2002).

In the human, ERα expression was localised to the efferent ducts in fetal and adult testes but no expression was seen in the Leydig cells, Sertoli cells, peritubular myoid

cells or the germ cells (Gaskell et al., 2003; Saunders et al., 2001). A comparison of the expression of ERα in the testes from the human, the macaque and marmoset monkey showed that the expression of ERα in both primates was identical to that in the human with the protein shown to be localised to the efferent ducts but not in any of the cells of the testis (Saunders et al., 2001). In the case of the marmoset, this differs from the study by Fisher where immunopositive Leydig cells were observed (Fisher et al., 1997). From all these reports and others published, it is clear that the efferent ducts are a major site for oestrogen action as the expression of ERα is seen across numerous species (Hess, 2003). Further evidence for this has been revealed by studies in ERα knockout mice (section 1.9.1.1).

1.8.1.2. Oestrogen receptor beta (ERβ)

ERβ has been cloned from various species including mouse, human, primate, fish and eel (Enmark et al., 1997; Mosselman et al., 1996; Todo et al., 1996; Tremblay et al., 1997). Analysis of the tissue distribution of ERβ has revealed differences in expression between species.

In the female, the expression of ERβ is seen throughout the reproductive tract in various species. In the rat, the study by Saunders et al. (1997) localised ERβ protein to the granulosa cells and the corpora lutea of the ovary and throughout the oviduct epithelium. In the rat uterus, ERβ was seen in the epithelium and in the stroma (Saunders et al., 1997). In the human, ERβ is expressed in the granulosa cells of ovary and ERβ has been localised to the uterus at all stages of the menstrual cycle (Critchley et al., 2001; Saunders and Critchley, 2002). ERβ is also expressed throughout the marmoset ovary (Saunders et al., 2000).

In the male, ERβ is expressed in multiple cell types within the testis of various species as well as in the prostate and the epididymis (Couse and Korach, 1999; Couse et al., 1997; Enmark et al., 1997; Kuiper et al., 1997). ERβ was localised to the testis of the mouse by immunohistochemistry and was seen to be expressed in the Sertoli, Leydig, peritubular myoid and the germ cells (Zhou et al., 2002). In the male

rat, ERβ was expressed in the Sertoli cells, Leydig cells and some germ cells including spermatocytes and spermatogonia (Saunders et al., 1998) and this was confirmed in the study by Saunders et al. in 2001 where the expression of ERβ was compared between humans, macaque and marmoset testes and was found to be present in all three cell types of the testis. Expression of ERβ was greater in the Sertoli cells than in the germ cells in the marmoset, rat and the human (Saunders et al., 2001).

The expression of ERβ has also been described in the male reproductive tract of dogs and cats. In the dog, ERβ is expressed the germ cells and the peritubular myoid cells but no expression is seen in the Sertoli cells or the interstitial cells. In the cat, the expression is again seen in the germ cells and in the peritubular cells but not in Sertoli cells (Nie et al., 2002).

1.8.1.3. Androgen receptor

In the adult mouse, AR is expressed in the Leydig, Sertoli and peritubular myoid cells of the testis as well as throughout the epididymis, vas deferens and the efferent ducts. AR is not expressed in the germ cells. Similarly, in the adult rat testis, AR expression occurs in a stage specific pattern in the Sertoli cells and in the Leydig and peritubular myoid cells but not in the germ cells, AR staining in rat Sertoli cells appears to be dependant on the cycle of the seminiferous epithelium (Bremner et al., 1994). In humans, AR is expressed in the somatic cells of the testis but is absent from the germ cells. Sertoli cell expression of AR is again stage specific in the human (Suarez-Quian et al., 1999). In humans, mutations in the AR cause a diverse range of clinical conditions, including motor neuron disease (Kennedy's disease) and testicular feminization mutation (Tfm) syndrome (Choong and Wilson, 1998; Quigley et al., 1995).

1.9. Mouse knockout models

Mouse models with targeted disruption of the genes encoding both ER's, AR and the aromatase enzyme have been described (Dupont et al., 2000; Eddy et al., 1996; Krege et al., 1998; Lubahn et al., 1993; Yeh et al., 2002). The knockout mouse

models also show that oestrogens and androgens are essential for fertility but not for survival after birth or for the formation of the reproductive tract.

1.9.1. Oestrogen receptor knockout mice

1.9.1.1. Oestrogen receptor α knockout mice (ERKO)

The role of the ERα in the reproductive tract was investigated by the generation of a loss of function mouse model. ERKO mice were generated by targeted insertion of a 1.8kb fragment into exon 2 of the ERα gene, disrupting the DNA binding domain of the gene (Lubahn et al., 1993). A second line of ERKO mice has been generated by Dupont et al. (2000). Female mice ERKO were found to be infertile. Histological analysis of the reproductive tract showed abnormal uteri and cystic ovaries with few granulosa cells and no corpora lutea (Dupont et al., 2000; Lubahn et al., 1993).

At birth, male ERKO mice appear normal, however adult males are infertile, consistent with an essential role for ERα-mediated processes in the regulation of male reproduction. Adult ERKO male mice have significantly fewer epididymal sperm than heterozygous or wild-type males, the motility of the sperm is reduced (Couse and Korach, 1999; Eddy et al., 1996) and the testes begin to degenerate and become atrophic (Hess et al., 1997). Spermatogenesis can be observed in some seminiferous tubules but most tubules have dilated lumens and a disorganized seminiferous epithelium. This disruption of spermatogenesis and degeneration of the seminiferous tubules becomes apparent after 10 weeks of age. The sperm also have reduced motility and are unable to fertilize eggs in vitro (Eddy et al., 1996; Mahato et al., 2001). The ERKO mice created by Dupont et al. (2000) showed a similar phenotype. The mice were also infertile showing a loss of germ cells and dilated tubules (Dupont et al., 2000).

The efferent ducts appear swollen in ERKO males and the lumen of the duct is severely dilated (Hess et al., 1997). As previously discussed in this chapter, the efferent ducts express the highest levels of ERα in the male reproductive tract in various species. The ducts are involved in sperm transport from the tubule to the

epididymis and play a role in fluid reabsorbtion with the ducts reabsorbing approximately 90% of the fluid from the rete testis (Hess, 2000; Hess et al., 1997). This reabsorbtion acts to concentrate the spermatozoa as they enter the epididymis resulting in maximum spermatozoa number being released per ejaculation (Ilio and Hess, 1994). In the efferent ducts of the ERKO mice, the height of the microvillus border in the epithelial cells is reduced and this affects fluid resorption (Hess et al., 2001). This knowledge along with the finding that the efferent ducts are swollen due to accumulation of luminal fluid has led to the suggestion that one of the main roles played by ERα in the male is in mediating fluid reabsorbtion and that disruption of this process was the cause of infertility in the ERKO male mice (Hess et al., 1997).

1.9.1.2. Oestrogen receptor β knockout mice (βERKO)

The first male mice with targeted disruption of ERβ were produced by targeted insertion of a 1.8kb fragment into exon 3 of ERβ (Krege et al., 1998). Dupont et al. (2000) used a similar targeting strategy to generate a second βERKO line (Dupont et al., 2000). Both male and female mice develop normally and young animals are grossly normal. Adult female βERKO mice have reduced fertility compared to wild-type littermates producing fewer and smaller litters. This reduction in fertility is due to a reduction in ovarian function with more atretic follicles and less corpora lutea being found in the βERKO animals. Sexual behaviour was normal in the knockouts and mammary gland development and function was normal (Krege et al., 1998).

Male βERKO mice are fertile and show no gross abnormalities in the reproductive tract (Couse and Korach, 1999; Krege et al., 1998). However, data exists showing cellular changes in the prostate with ERβ knockout mice developing severe epithelial hyperplasia in some ducts of ventral prostate as the mice age (Weihua et al., 2001; Weihua et al., 2002) but this has been disputed by other studies (Dupont et al., 2000) and the role of ERβ in the prostate is unclear.

ERβ is expressed in bone, suggesting oestrogen action on the skeleton is important in both men and women (Braidman et al., 2001). It has been shown that men lacking

aromatase suffer from osteopenia (Morishima et al., 1995) and bone loss in ageing men is associated with a reduction in the levels of oestrogens (Turner et al., 1994). In postmenopausal women, a reduction in the levels of oestrogens is also associated with bone loss and increased risk of osteoporosis.

No patients have yet been described lacking the ERβ gene but patients have been described that show polymorphisms within the gene resulting in menstrual disorders (Ogawa et al., 2000a; Sundarrajan et al., 2001). This information combined with the results from the ERα knockout mice (section 1.9.1.1) and the double knockout ERαβKO mice suggest that the primary role of ERβ is to modulate gene regulation via ERα.

1.9.1.3. α / β ERKO mice

ERα and ERβ have both differential and overlapping distribution throughout the male reproductive tracts (Couse et al., 1999) and differences also exist in the transcriptional activities between the two receptors (Paech et al., 1997). Mice lacking functional ERα and ERβ receptors have been generated by crossbreeding the ERKO and βERKO mice (Couse et al., 1999; Dupont et al., 2000). ERαβKO male mice possess a grossly normal reproductive tract, but are infertile. The testes of adult ERαβKO males had some residual spermatogenesis but the number and the motility of sperm were reduced. Adult males showed a loss of germ cells and the rete testis appeared dilated (Dupont et al., 2000). This phenotype is similar to that of the ERKO male and is characteristic of a loss of the ERα gene.

ERαβKO females exhibit normal differentiation of the uterus, cervix, and upper vagina. Uterine hypoplasia is seen in ERαβKO adult females, which resembles that in the ERKO mice but this does not occur in ERβKO females. This suggests differentiation of the female reproductive tract during fetal life can occur in the absence of a functional ERα and ERβ. Ovaries of adult ERβKO females exhibited primordial and growing follicles but no corpora lutea were observed. The ovaries of adult ERαβKO mice contained structures that resembled the seminiferous tubules of

the testis and lacked granulosa cells. In some, a degenerating oocyte was present, whereas others showed no evidence of germ cells. (Couse et al., 1999; Dupont et al., 2000).

1.9.2. Androgen receptor knockout mice

The testicular feminisation mouse (Tfm) has a single basepair deletion in the AR gene which results in a frameshift mutation and translation of a truncated protein which lacks both the DNA binding and ligand binding domains (Charest et al., 1991). Tfm mice are totally insensitive to androgens and hence have been used to study the role of the androgen receptor in normal development (Murphy and O'Shaughnessy, 1991). AR function is essential for masculinisation during fetal development. Tfm mice have a grossly abnormal male reproductive tract with the testes failing to descend at the normal time (25 days) and are completely sterile (Murphy et al., 1994). Adult Tfm testes have normal Leydig cell numbers but the levels of androgens produced is severely reduced due to a loss of the enzyme 17α-hydroxylase (Murphy and O'Shaughnessy, 1991).

Gene targeting via the use of the cre-lox system (Holt and May, 1993) has been used to perform specific deletion of the AR (Yeh et al., 2002). AR knockout (ARKO) male mice exhibit the same phenotype as the Tfm mice with a feminised appearance and small cryptorchid testes. The mice also exhibit signs of micropenis and hypospadias. Immunohistochemical evaluation of the testis showed that no AR staining was present in 8-week-old mice. Histological analysis of the testes revealed that spermatogenesis was incomplete with only a few germ cells being found in the tubules. No round or elongated spermatids or sperm was seen in the tubules. Sertoli cells and Leydig cells showed signs of degeneration and hypertrophy and the number of Leydig cells was reduced, suggesting that androgens exert their actions on spermatogenesis via Sertoli cells (Yeh et al., 2002).

The Tfm/ARKO mice phenotypes may be partly due caused or compounded by the cryptorchidism shown by these mice. A way to overcome this has become available with the recent generation of Sertoli cell selective androgen receptor knockouts using

cre-lox strategies (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004). In the study by De Gendt et al. (2004), the authors created a ubiquitous AR knockout mouse as well as a selective knockout with AR absent from the Sertoli cells only (SCARKO) to further define the role of the Sertoli cell as a target for androgens. (De Gendt et al., 2004). The ARKO mice created showed a similar phenotype to other ARKO mice described above, displaying complete androgen insensitivity syndrome. The testes were smaller and positioned incorrectly within the abdominal or inguinal region in males. In the female mice, no uterus or fallopian tubes were observed (De Gendt et al., 2004).

Sertoli cell selective deletion of AR was achieved by cross breeding mice with a floxed AR gene with mice expressing cre-recombinase under the control of the AMH promoter. To date, three lines of SCARKO mice have been generated and in all cases exhibit normal external sexual development with the testes being correctly positioned in the males overcoming the issue of a compounded phenotype due to cryptorchidism. A difference was observed in testis size however with the SCARKO mice showing a 28.4% reduction in testis size compared to the wild type mice. Epididymis weight was also reduced by 30% but the mice showed normal development of the rest of the male reproductive tract including the prostate, seminal vesicles and epididymis. Upon histological analysis, spermatogenesis was incomplete. The number of germ cells was reduced with a loss of pachytene and primary spermatocytes. Few secondary spermatocytes and round spermatids were observed but no elongate spermatids were seen in the tubules. These observations were confirmed by quantitative PCR analysis showing a lack of protamine 1 and 2 expression in the SCARKO testes. Immunohistochemistry looking for AR expression in the SCARKO testes showed the Leydig cells and peritubular cells were positive for AR but no AR protein expression was seen in any Sertoli cell (De Gendt et al., 2004).

The Sertoli cell specific AR knockout mouse model described by Chang et al. (2004) also showed a reduction in testicular size. In their knockout, termed S-AR-/y, spermatogenic arrest was also observed, determined by flow cytometric scanning of

propidium iodide-labelled cells from the S-AR-/y testis and results from this suggested that the spermatogenic arrest occurred in S-AR-/y mice before the first meiosis (Chang et al., 2004). Conclusions drawn from the investigation of another Sertoli cell selective knockout generated by Holdcraft and Braun (2004) were that Sertoli cell AR expression is not required for the completion of meiosis or the differentiation of round spermatids prior to elongation and the primary role of AR function is in the final stages of spermiation and is required for the terminal differentiation of sperm and the release of mature sperm into the lumen of the tubule (Holdcraft and Braun, 2004). This model however exhibits a hypermorphic allele and exhibits a slightly different phenotype from the other two models.

These mouse models along with the earlier evidence from the ARKO mice have determined that a functional AR is a requirement for the maintenance of normal spermatogenesis and is essential for the germ cells to be able to successfully complete meiosis (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004).

1.9.3. Aromatase knockout mice (ArKO)

Cytochrome P450 aromatase is the product of the CYP19 gene. Fisher et al. (1998) have generated mice lacking a functional aromatase enzyme (ArKO) by targeted disruption of exon 10 in the *cyp19* gene. By disrupting aromatase activity, oestrogen biosynthesis is blocked and oestrogens are therefore not available to interact with ERα and ERβ. Knockout mice with a disrupted aromatase gene (ArKO) are born phenotypically normal. At sexual maturity, female ArKO mice begin to masculinise. Male ArKO mice appear to be fertile when young and the gross morphology of the testes in the younger males appear normal. Fertility was compromised as the mice aged, and litter sizes are reduced. By 18 weeks, one out of five males show disrupted spermatogenesis with degeneration of the round spermatids and a lack of elongated spermatids. (Fisher et al., 1998; Murata et al., 2002; Robertson et al., 1999). Male ArKO mice had elevated testosterone and LH levels but FSH levels were normal. Another ArKO mouse model reported that only 14% of males were fertile (Toda et al., 2001) and a further line generated by Honda et al. (1998) reported total infertility

in the knockouts as well as showing a behavioural phenotype, with reduced aggressiveness in the males and impaired sexual behaviour (Honda et al., 1998). The ArKO mice exhibit some similarities to the ERαβKO mice described in section 1.9.1.3 with the females of both knockout lines exhibiting seminiferous tubule like structures within the ovaries however, there is no similarity seen in the male phenotype as the ArKO mice do not show any dilation of the efferent ductules or any loss of germ cell numbers (Couse et al., 1999; Dupont et al., 2000; Fisher et al., 1998).

P450 aromatase is encoded for by the CYP19 gene and in man, patients with defects in the CYP19 gene exist (Simpson et al., 1997) resulting in problems in bone formation highlighting an important role in bone metabolism in men. This phenotype in the bone can be alleviated by administration of oestrogens (Simpson et al., 1997). Consistent with the reports that oestrogens play a role in bone cell function (Braidman et al., 2000; de Ronde et al., 2003), and in the cardiovascular system (Gray et al., 2001), the ArKO mice exhibit a decrease in bone volume and thickness and increased bone resorption (Miyaura et al., 2001; Oz et al., 2000) and an accumulation of adipose tissue associated with hypercholesterolemia and insulin resistance (Jones et al., 2000).

1.9.4. Oestrogen sulfotransferase knockout mice

Oestrogen sulfotransferase (EST) is a cytosolic enzyme that catalyses the sulfation and inactivation of oestrogens (Strott, 1996; Tong et al., 2004). It is known that although oestrogens are synthesised in the testis, as shown by the presence of the aromatase complex, uncontrolled oestrogen activity in the testis leads to inhibition of steroidogenesis and spermatogenesis in mice (O'Donnell et al., 2001; Tong and Song, 2002). EST is highly expressed in the Leydig cells and in the middle to distal regions of the epididymis of male mice (Tong and Song, 2002). EST knockout mice were indistinguishable from wild type littermates at 2 months. However, as the animals aged, the knockout mice began to show abnormalities in the testis compared to the control animals. By age 12 months, the Leydig cells of the knockouts were hyperplastic and damage to the seminiferous tubules was observed with the tubules

being filled with large vacuoles and the seminiferous epithelium disrupted. A significant decrease in testis weight and a reduction in sperm motility of up to 80% was also seen in the knockout animals (Qian et al., 2001). EST is not expressed in the fetal and immature testis and as the fetal development and sexual determination is normal in the knockouts, it is thought that the damaging effect of a lack of EST in the testis is a progressive and age dependant result of altered paracrine stimulation due to unmetabolised oestrogen (Qian et al., 2001).

1.9.5. Follicle stimulating hormone knockout mice

FSH acting via its receptor which is expressed in Sertoli cells (Sairam and Krishnamurthy, 2001) is thought to play a major role in the regulation of spermatogenesis. Further evidence of the role(s) played by FSH have been obtained from mouse models involving targeted deletion of FSH and the FSH receptor (Abel et al., 2000; Dierich et al., 1998; Kumar et al., 1997).

1.9.5.1. FSHβKO mice

Removal of the β subunit of FSH results in female mice that are infertile due to a block in folliculogenesis. FSHβKO males are fertile but have reduced testis sizes and a reduction in sperm motility (Kumar et al., 1997; Sairam and Krishnamurthy, 2001). Circulating testosterone levels were reported to be normal in the adult FSHβKO mice and Leydig cell number and function were normal in the knockouts. In a study using stereological analysis, Wreford et al. (2001) claimed that the defects seen in the FSHβKO knockout mice are related to diminished FSH action resulting in a reduction in Sertoli cell proliferation and the capacity of Sertoli cells to nurture germ cells (Wreford et al., 2001).

1.9.5.2. FSHRKO mice

The FSH receptor (FSH-R) is a G-protein–coupled, seven transmembrane receptor and is expressed in the Sertoli cells of the male and the granulosa cells of the female. Generation of mice carrying a null mutation of the FSH-R resulted in females that were infertile. In two separate lines of mice, ovary size was significantly reduced and

folliculogenesis was arrested at the pre-antral stage (Abel et al., 2000; Dierich et al., 1998).

However, the two different lines of FSH-R knockout mice described exhibit differences in the phenotypes of the males. The FSHRKO generated by Dierich et al. (1998) resulted in male mice with small testes, partial spermatogenic failure, and reduced fertility. Testosterone levels were reduced in by 50% but this was still enough to maintain partial spermatogenesis (Dierich et al., 1998). This was a more severe phenotype than the one described for the FSHRKO mice generated by Abel et al. (2000), where the male mice were fully fertile although they did show a reduction in testicular weight and the gonadotrophin feedback mechanisms were disturbed resulting in altered hormonal levels (Abel et al., 2000). Although differences in the two knockouts exist, it is clear that FSH signalling is not essential for initiating spermatogenesis, but it is thought to be involved in the maintenance of viability and motility of sperm.

A recent study investigating the effect of the absence of the FSH-R on Sertoli cell function reported a reduction in ABP expression and analysis by electron microscopy showed large spaces within the Sertoli cells that are believed to be an accumulation of fluid in the Sertoli cell cytoplasm (Grover et al., 2004). Jegou et al. (1982) proposed that FSH is involved in fluid absorption and/or secretion and it is known that Sertoli cells are involved in fluid transport from the interstitial space into the lumen of the seminiferous tubule to aid in sperm movement from the testis to the epididymis so it is not surprising that this phenotype exists in the FSHRKO mice (Jegou et al., 1982).

Mice lacking the FSH-R also appear to have altered Leydig cell function. Baker et al. (2003) reported that the lack of FSH-R resulted in a reduction in the number of Leydig cells in adult animals (Baker et al., 2003b). This reduction in the number of Leydig cells could account for the drop in testosterone levels observed by Dierich (1998) but this does not seem to occur in the FSHβKO males (Abel et al., 2000).

1.9.6. Luteinising hormone receptor knockout mice (LuRKO)

The LH receptor is expressed in interstitial Leydig cells. Mice that lack the LH receptor appear normal at birth with both testicular size and location similar to that of wild-type littermates (Zhang et al., 2001). This is consistent with gonadotrophin-independent production of testosterone by the fetal Leydig cells. Testosterone production in the adult testis is regulated by LH. In the fetal testis, the regulation of testosterone production is unclear and it is has been suggested that the onset of androgen production in the fetal testis takes place in an LH-independent way (Majdic et al., 1998). By age 30-35 days, knockout males exhibit an undeveloped scrotum and micropenis. By 7 weeks of age, the males have very small testis compared to wild types and show signs of abnormal spermatogenesis. The seminiferous tubules are poorly developed and spermatogenesis is arrested at the round spermatid stage. Female mice show a similar pattern of events, they are born phenotypically normal but by age 7 weeks, ovarian size was decreased and follicles only progressed to the early antral stage. The main role of LH is to simulate testosterone synthesis by the Leydig cells (Cooke and Saunders, 2002), in the LuRKO mice, Leydig cell size and number was dramatically decreased were poorly differentiated due to their inability to respond to LH. The male testicular phenotype is reminiscent of that seen in the SCARKO males (De Gendt et al., 2004) and would be consistent with inadequate production of testosterone by the Leydig cell population.

1.10. RNA interference

Many scientific advances have been achieved by the use of transgenic and knockout animals, however, these animals are costly and time consuming to create. The advent of a new technology, termed RNA interference (Fire et al., 1998), has the potential to overcome some of the problems associated with the generation of knockout mice, allowing knockdown of specific genes in mammalian systems.

1.10.1.Overview of the method

RNA interference is the process of sequence specific post transcriptional gene silencing initiated by double stranded RNA (dsRNA) (Elbashir et al., 2001). The process has been observed in a variety of organisms including plants, fungi, insects,

protozoans and mammals (Bernstein et al., 2001; Elbashir et al., 2001; Harborth et al., 2001; Moss, 2001). Since its discovery in *C. elegans* (Fire et al., 1998), RNAi has been developed as an important tool in the study of gene expression. The uptake of dsRNA had been shown to be effective in silencing specific genes in insects and plants but this method initially proved unsuccessful in mammals. When long double stranded RNA's, typically >200 nucleotides are introduced into cells, they get processed into 20-25 nucleotide small interfering RNA's (siRNA's) by an enzyme complex called Dicer. Dicer, discovered in *Drosophila* (Bernstein et al., 2001) contains domains for dsRNA binding, RNA unwinding and ribonuclease activity (Denli and Hannon, 2003). The siRNA's then assemble into endoribonuclease-containing complexes known as RNA-Induced Silencing Complexes (RISC). The siRNA guides the RISCs to the complementary RNA where they cleave the RNA Figure 1.11 shows the mechanism of the RNAi process.

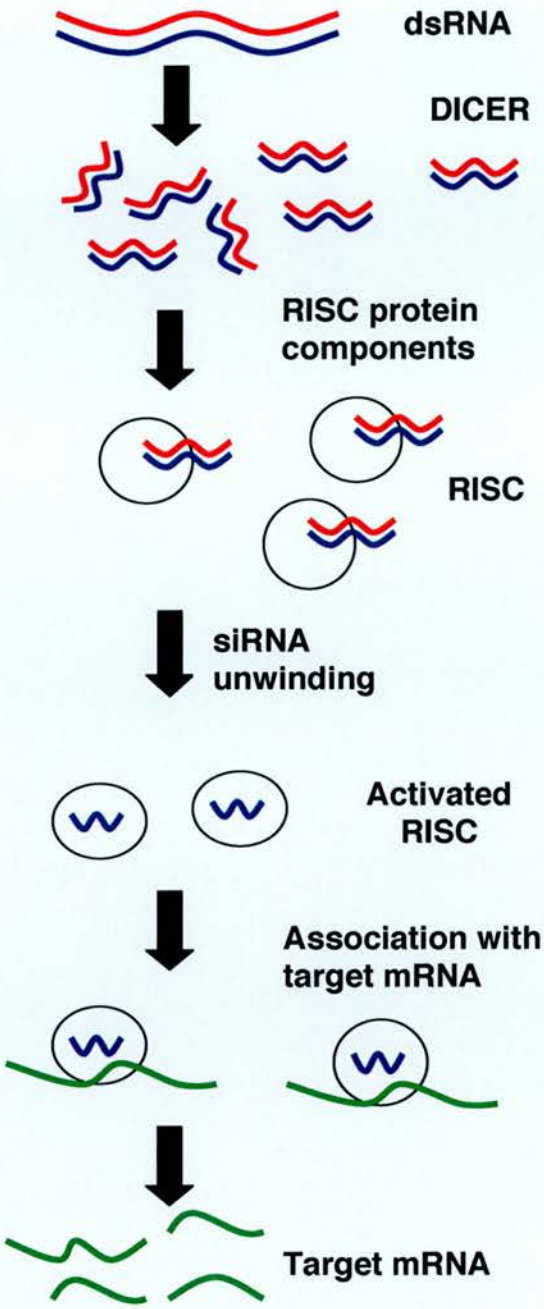


Figure 1.11 The mechanism of RNA interference, adapted from Ambion (ww.Ambion.com).

1.10.2.Mammalian siRNA

Introduction of dsRNA into the cytoplasm of mammalian cells triggered a number of pathways that mediated suppression of gene expression some of which were non-specific and included global inhibition of protein synthesis and non-sequence specific degradation of mRNA. Furthermore, dsRNA acted as a potent inducer of type 1 interferon synthesis and activated two classes of IFN induced enzymes: PKR, which is a dsRNA dependent protein kinase and 2',5' oligoadenylate synthetases (Sledz et al., 2003). Because the 2'5 oligoadenylate synthase activates RNaseI, which leads to mRNA cleavage, and PKR phosphorylates the translation initiation factor eI2α, which leads to inhibition of mRNA translation, activation of the interferon system had the effect of shutting down protein synthesis. In 2001, Elbashir et al. showed for the first time that chemically synthesised 21 nucleotide small interfering RNA's (siRNA's) could mediate knockdown of specific mRNAs in Hek293 and HeLa cells. (Elbashir et al., 2001) and that the interferon response was not initiated. It is thought that the short size of the siRNA's mimic's the products of the Dicer enzyme and avoids the PKR response (Paddison et al., 2002). After transfection with siRNA's, a reduction in specific mRNA can be detected within 24 hours and the effect can last for several days depending on the turnover rate of the RNA or protein.

1.10.3.Vector delivery systems

Although heralded as the scientific breakthrough of the year in 2002, RNAi is not without problems. Working with RNA can be problematic and degradation of siRNA's is common. Also, the knockdown effect of the siRNA is transient so long term effects of gene silencing cannot be observed. The advent of RNAi vectors, especially short hairpin RNA's (shRNAs) in 2002, (Brummelkamp et al., 2002) was an important technical advance and allowed more stable expression of the siRNA whilst working with a DNA based vector. Most short hairpin vectors contain the RNA polymerase III H1 promoter, resulting in a small RNA transcript with a well-defined transcription start site and a 5-thymidine RNA termination site. Other vectors have been successfully used containing the Pol III U6 promoter. In these vectors, the target gene specific insert contains the siRNA 19nt followed by a short (9nt) spacer

sequence then the reverse complementary 19nt siRNA which folds into a hairpin loop structure (Brummelkamp et al., 2002). This is shown in Figure 1.12. When the vector is expressed in mammalian cells, the hairpin loop containing the siRNA sequence is recognised by Dicer and is cleaved to form a functional siRNA (Brummelkamp et al., 2002).

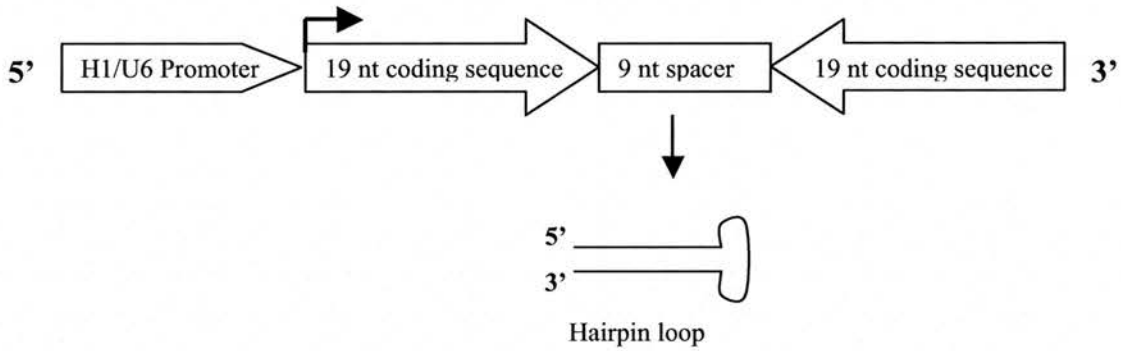


Figure 1.12 siRNA synthesised from a DNA template.

Viral vectors containing siRNA's have also been developed which should allow an increase in the range of cells that can be transfected e.g. primary cells and these are also suitable for use *in vivo*. For example, Tiscornia et al. have designed a lentiviral vector capable of expressing siRNA in mammalian systems (Tiscornia et al., 2003). The siRNA construct with a H1 promoter was cloned into the 3' LTR of the lentivirus. Knockdown of GFP was achieved in 293T cells and successful transduction of preimplantation mouse embryos was shown. This decrease in GFP expression was also shown in the progeny derived from these embryos meaning that generation of "knockdown" mice through viral transgenesis was now achievable

1.11. Aims of the project

The aims of this study were to investigate the role of steroid hormones, in particular oestrogens in murine spermatogenesis. A major focus of these investigations was the role played by ERβ in the modulation of germ cell and somatic cell function. Initial studies focussed on the spermatogonial stem cells, which were successfully isolated and characterised. The impact of steroids on Sertoli cell function made use of a transformed murine cell line, which had maintained a differentiated Sertoli cell phenotype. Steroid receptor status, steroid responsiveness and the impact of targeted deletion of receptors using RNAi were all assessed.

Chapter 2

General Materials and Methods

2.1. Animal experimentation

Animal experimentation was carried out in accordance with the UK Home Office Animal Experimentation (Scientific Procedures) Act 1967 under project licence PPL60/2923. The appropriate ethics committee also approved all experiments. Wild type mice from heterozygote mating of the transgenic strain *Dazl* $^{Tm1hgu/m1hgu}$ (Ruggiu et al., 1997) were used for these studies and the mice were housed under standard conditions and fed *ad libitum.*

2.1.1. Tissue collection and fixation

Animals were killed by cervical dislocation or other approved Schedule 1 method. Tissues were removed from both immature (day 0 to day 21 animals) and adult mice. Tissues were fixed or snap frozen in liquid nitrogen for subsequent RNA extraction (section 2.5).

Fixation was carried out for 6 hours using Bouins solution (BIOS), which forms cross-links between proteins and aldehydes, resulting in a stable structure without loss or damage to antigenic sites. Samples were then transferred into 70% ethanol and processed into paraffin wax (section 2.2.1).

2.2. Immunohistochemistry

Immunohistochemistry is a technique which allows specific localisation of proteins within tissue sections and cultured cells using antibody–antigen interactions. The antigen is recognised within a cell using a specific antibody raised to an epitope of the protein in question.

2.2.1. Tissue processing and sectioning

Tissues were embedded in paraffin wax blocks by the histology support service, MRC Human Reproductive Sciences Unit. Wax blocks were cut into 5μm sections on a hand-operated microtome RM 2135 (Leica). The sections were floated onto ice cold followed by warm (42°C) distilled water and placed onto charged slides (Superfrost® Plus, BDH) and dried overnight at 50°C. The slides were de-waxed in xylene for 5 minutes then rehydrated through a series of graded alcohols (100%→90%→80%→70%) for 20 seconds in each alcohol and finally rinsed in water.

2.2.2. Antigen retrieval

Antigen retrieval was performed to reduce cross-linking of proteins formed during the fixation process, allowing the antibody to bind to the antigen (Shi et al., 1993). This was carried out by pressure-cooking. Slides were heated in 0.01M citrate buffer pH6 in a Tefal Clipso pressure cooker (Tefal). Briefly, the slides were placed in 2L of boiling citrate buffer; the lid was sealed and set to pressure setting 2. The slides were heated until full pressure was reached and left for 5 minutes. After this time, the pressure was released, the pressure cooker removed from the heat and the slides left in the hot buffer for 20 minutes before cooling with H_20 and transferring to TBS (section 2.13.1).

2.2.3. Methanol / peroxide block

Slides were incubated in 3% hydrogen peroxide (BDH) in methanol for 30 minutes to decrease endogenous peroxidase activity in an attempt to eliminate background staining. After blocking, slides were rinsed in tap water and washed three times in TBS, 5 minutes each wash.

2.2.4. Avidin-biotin block

The Avidin–Biotin blocking kit (Vector SP-2001) was used to block endogenous biotin, which can lead to non-specific staining. Four drops of avidin/ml of 1 part blocking serum (Diagnostics Scotland) / 4 parts TBS / 5% BSA (Sigma) were added to the slides and incubated for 30 minutes at room temperature. The slides were

washed in TBS three times for 5 minutes before biotin was added (4 drops per ml of TBS), incubated at room temperature for 15 minutes followed by 3 x 5 minute washes in TBS.

2.2.5. Primary antibodies

The appropriate primary antibody (Table 2.1) was diluted as required in blocking serum/TBS/BSA, applied to the slides and incubated overnight at 4°C.

Antigen	Dilution	Antigen retrieval	Species raised in	Source
ERβ	1:1500	Citrate	Sheep	Raised in house, (Saunders et al., 2000)
c-kit	1:30	Not required	Rabbit	Santa Cruz
RBM	1:500	Citrate	Rabbit	Raised in house
AR	1:200	Not required	Rabbit	Santa Cruz
Oct-4	1:75	Citrate	Goat	Santa Cruz
GATA-1	1:100	Not required	Goat	Santa Cruz

Table 2.1 Details of primary antibodies.

2.2.6. Secondary antibodies

After incubation with the primary antibody, the slides were washed in TBS three times for 5 minutes. The appropriate secondary antibody (Table 2.2) was diluted in blocking serum, added to the slides and incubated for 1 hour at room temperature and washed off with TBS as before.

Antibody	Dilution	Source
Rabbit anti mouse biotinylated	1:500	DAKO
Swine anti rabbit biotinylated	1:500	DAKO
Rabbit anti sheep biotinylated	1:500	Vector
Rabbit anti goat biotinylated	1:500	Vector

Table 2.2 Details of Secondary antibodies.

2.2.7. Horse-radish peroxidase

The ABC-HRP detection system (DAKO) was used to amplify the signal obtained from the secondary antibody. Avidin is composed of four subunits, which form a tertiary structure possessing four biotin-binding pockets. The ABC-HRP complex works because 3 of the biotin binding sites on the avidin molecule are occupied with biotin molecules complexed to HRP whilst one site remains empty and can bind to the biotinylated secondary antibody (Figure 2.1).

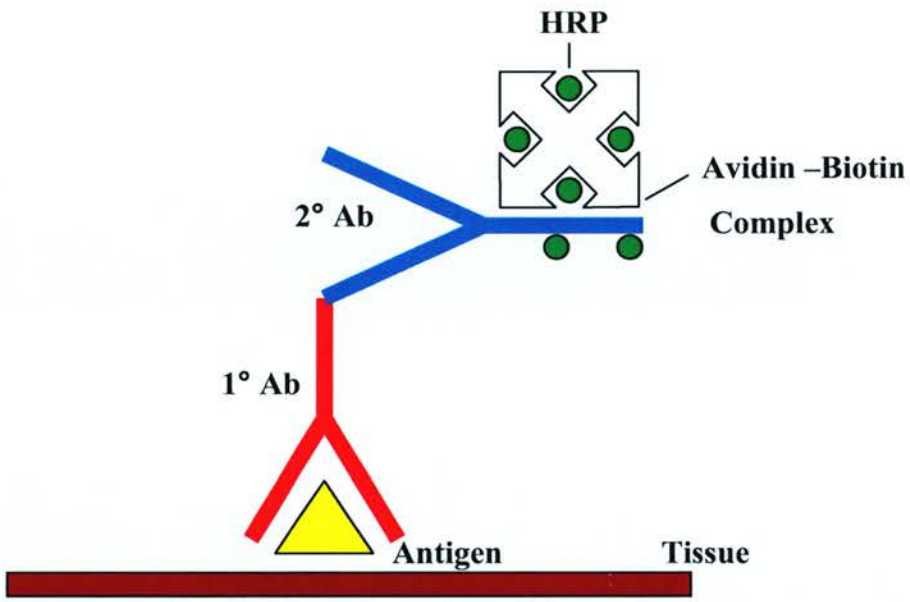


Figure 2.1 ABC-HRP reaction.

ABC-HRP was prepared by adding 1-drop solution A (DAKO) and 1-drop solution B (DAKO) to 5 ml of Tris-HCl. This was made up 20 minutes prior to use. ABC-HRP was added to the slides for 30 minutes at room temperature followed by 3 x 5 minute washes in TBS.

2.2.8. Developing, counterstaining and mounting

The slides were developed using diaminobenzidene (DAB); 1 drop/ml in DAB buffer (DAKO). After DAB staining, the slides were counterstained with haematoxylin, differentiated in acid-alcohol and the blue nuclei developed in Scott's tap water. The slides were dehydrated in increasing concentrations of alcohol and cleared in

histoclear and xylene for 5 minutes each before being mounted under a glass coverslip using Pertex mounting medium (CellPath).

2.2.9. Immunohistochemistry on cultured cells

Cells plated out on 2 well or 8 well glass chamber well slides (Nunc) at the appropriate density (Table 2.4) to be stained were fixed in 100% ice cold methanol or modified Davidson's (Section 2.13.1) fixative (for fluorescent immunohistochemistry) for 10 minutes. The fixative was removed and the cells were washed in PBS. The cells were permeablised with the addition of 0.2% NP40 (Sigma), 1% BSA (Sigma) 10% appropriate blocking serum (Diagnostics Scotland) in TBS for 20 minutes at room temperature. The cells were washed twice in TBS and the procedure for immunohistochemistry for tissue sections from the avidin-biotin blocking step (section 2.2.4) was followed.

2.2.10. Fluorescent immunohistochemistry

Fluorescent immunohistochemistry was carried out on cultured cells. Cells were fixed in modified Davidson's fixative (Section 2.13.1) for 10 minutes at room temperature followed by 2 washes in PBS for 5 minutes each wash. Non specific staining was blocked by addition of 1 part blocking serum (Diagnostics Scotland)/ 4 parts PBS / 5% BSA (Sigma) for 30 minutes at room temperature then washed twice in PBS for 5 minutes each wash. Primary antibody (Table 2.3) was added and the cells were incubated overnight at 4°C. The following day, the cells were washed twice for 5 minutes each wash in PBS before addition of the appropriate fluorescently labelled secondary antibody (Table 2.3) and incubated for 1 hour at room temperature. The cells were washed twice in PBS and then counterstained with either To-Pro-3 or propidium iodide to visualise the nuclei. To-Pro-3 (Molecular Probes) was added at a 1:2000 dilution for 2 minutes before a final PBS wash. Propidium iodide (Sigma) was added at a concentration of 1:2000 for 2 minutes before a final PBS wash. The slides were mounted under a glass coverslip using Permafluor mounting medium (Immunotech) and observed using the confocal microscope (section 2.11.4).

Primary antibody	Dilution	Species raised in	Supplier
Tyrosine α- tubulin	1:2000	Mouse	Sigma
β-tubulin	1:1500	Mouse	Sigma
Lamin A/C	1:500	Mouse	Santa-Cruz
Secondary antibody	**Dilution**	**Species raised in**	**Supplier**
Goat anti-mouse Alexa fluor 546	1:200	Goat	Molecular probes
Goat anti-mouse Cy-5	1:60	Goat	Molecular probes

Table 2.3 Details of primary and fluorescently labelled secondary antibodies used on cultured cells.

2.2.11. Imaging

Slides were examined using an Olympus Provis microscope (Olympus Optical Co.) and images captured using a Kodak DCS330 camera (Eastman Kodak Co.). Captured images were compiled using Photoshop CS (Adobe).

2.3. Cell culture

Cell culture experiments were performed in a class II cabinet and the cells maintained in a humidified incubator either at 33°C, 37°C or 39°C with 5% CO_2.

2.3.1. Cell lines

SK11 cells were a gift from Drs Walther and Ivell, Hamburg; (Walther et al. 1996). The cell line was derived from Sertoli cells from a 10 day old H-2k^b –tsA58 transgenic mouse (Jat et al. 1991) and immortalised using the SV40 large T antigen. The cells remain mitotically active at 33°C where they exhibit characteristics of immature Sertoli cells but when cultured at the non-permissive temperature of 39°C, they differentiate and stop dividing.

GC-1 and GC-2 cells were obtained from ATCC. GC-1 cells (Hofmann 1992) are a germ cell line derived from testes of a 10-day-old BALB/c mouse were immortalised using the SV40 large T antigen. GC-2 cells (Hofmann and Millan, 1995) are derived from spermatocytes isolated from a 6-week-old BALB/c mouse transformed with the

SV40 large T antigen and also contain a temperature sensitive mutant of the p53 tumour suppressor gene (LTRp53cG9).

STO cells were purchased from ECACC. They are a mouse embryo fibroblast cell line that has been selected for 6-thioguanine and ouabain resistance. The cells were mitotically inactivated by treatment with Mitomycin C and used as a feeder layer for the culture of spermatogonial stem cells (Chapter 4, section 4.2.8.1).

2.3.2. Passaging of cells

Media was removed from the cells and discarded; cells were rinsed with 5 ml PBS (Sigma) to remove any traces of media. The PBS was removed and 1.5 ml 1 x Trypsin /EDTA (Gibco) added per T75 flask. The cells were incubated at 37°C for 3-5 minutes until they had detached from the surface of the flask; thereafter 8.5 ml "complete medium" (section 2.17.2) was added containing 10% FCS which halts any further action of the trypsin. If required, a cell count was performed using a haemocytometer before the cells were diluted and plated out in new flasks.

2.3.3. Transient transfection of cells

Cells to be transfected were trypsinised and plated out at the appropriate density for the culture vessel used (Table 2.4) the day before transfection. If required, the cells were also changed into Phenol-red-free media (section 2.13.2). Three different transfection reagents were routinely used for transient transfections: JetPEI, (Qbiogene, section 2.3.3.1) for luciferase reporter transfections; oligofectamine (Invitrogen) for siRNA transfections (section 2.11.3.1) and JetSI (Poly Transfection) (Chapter 3, section 3.2.8.3) for fluorescently labelled siRNA transfections. All transfections were performed in duplicate and carried out at least 3 times. At the time of transfection, cells were 70% confluent to increase transfection efficiency.

Culture vessel	Number of cells plated
8 well glass slides	1 x 10^4 cells / well
2 well glass slides	1 x 10^5 cells / well
12 well plate	1 x 10^5 cells / well
6 well plate	4 x 10^5 cells / well
3.5 cm glass bottomed dish	2 x 10^5 cells / dish

Table 2.4 Details of cell number plated onto various culture vessels.

One hour prior to transfection, the media on the cells was removed and replaced with an appropriate volume of serum free transfection media (section 2.13.2), which contained no antibiotics.

2.3.3.1. JetPEI

JetPEI (QBiogene) was used for luciferase transfections and volumes stated below are for transfections carried out in 12 well plates; for other plate sizes the volumes were scaled up or down accordingly. A total of 2μg DNA was diluted into 50μl 150mM NaCl, vortexed gently and centrifuged briefly. In a separate tube, 4μl JetPEI reagent was diluted in 50μl 150mM NaCl, vortexed and spun down. The JetPEI solution was immediately added to the DNA containing solution and the mixture vortexed then briefly centrifuged. The complex was incubated for 30 minutes at room temperature before being gently added to the cells in each well, which already contained 500μl serum free media. After 4 hours, incubation at 37°C, 5% CO_2, 500μl complete media with ligand (if required) was added and the cells were incubated for a further 24-48 hours.

2.3.4. Ligands

Various ligands were added during transfection to stimulate the cells: 17β-Oestradiol (E_2, Sigma), 5 alpha-androstane-3-beta, 17 beta-diol (3βAdiol, Sigma) both natural oestrogenic ligands, and diethylstilbestrol (DES, Sigma), a synthetic oestrogen were all reconstituted in 100% ethanol and serial dilutions (10^{-3}M to 10^{-11}M in ethanol) were made. Genistein (Sigma) which is classed as a phytoestrogen was reconstituted

in DMSO and serially diluted (10^{-3}M to 10^{-11}M in DMSO). Two receptor selective synthetic ligands, DPN™ and PPT™ were also used; 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN™, Tocris Cookson) is an ERβ agonist while 4,4',4"- (4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT™, Tocris Cookson) is a highly potent ERα agonist. Both of these ligands were reconstituted in 100% ethanol and serially diluted in ethanol as before. Testosterone (Sigma) was reconstituted in 100% ethanol and serial dilutions (10^{-3}M to 10^{-11}M in ethanol) were made.

2.4. Dual-Luciferase® Reporter gene assay

Reporter systems are used to study gene expression. Dual reporters are commonly used to improve experimental accuracy by measuring two individual reporter enzymes within a single system. The Promega Dual-Luciferase® reporter assay system measures the activity of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis,* also known as sea pansy) luciferases. In this study, both 3x ERE luciferase and pem-Luc reporter constructs were used. The 3x ERE-TK-Luc reporter construct was a gift from S.C. Nagel and D.P. McDonnell (Nagel et al., 2001). The pem-Luc construct was a gift from Professor G. Verhoeven and contains a functional ARE (Barbulescu et al., 2001). The pRL-CMV vector (Promega) contains the CMV enhancer and early promoter elements, which provide a high level expression of Renilla luciferase, which was used as the internal control. Because of their distinct evolutionary origins, both luciferases have dissimilar enzyme structures and substrate requirements. The experimental reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected control reporter provides an internal control. Normalising the activity of the experimental reporter to the activity of the internal control minimises experimental variability caused by differences in cell viability, transfection efficiency and pipetting errors. The results are presented as a fold increase in activity over the control.

2.4.1. Cell lysis

The media was removed from the cells and 250µl 1x Passive Lysis Buffer (Promega) added per well of the 12 well plate. The plate was incubated at room temperature for

15 minutes on a rocking platform. The cell lysate was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 14,000 rpm at 4°C for 30 seconds.

2.4.2. Reporter assay

All reagents used in the reporter assay were obtained from Promega.

Luciferase Assay Reagent II (LAR II) was prepared by resuspending the lyophilised Luciferase Assay Substrate in 10 ml of Luciferase Assay Buffer II. One volume of 50X Stop & Glo® Reagent was added to 50 volumes of Stop & Glo® Buffer and vortexed for 10 seconds.

The reporter assay was performed on a microplate luminometer (Berthold Laboratories). The luminometer was primed with LAR II. Cell lysates (20µl each) were pipetted in duplicate into a white 96 well plate (Nunc), the luminometer was programmed to dispense 30µl of LAR II reagent into each well and perform a 10 second measurement. Once the entire plate was read, the firefly luciferase measurements were saved and the luminometer washed with water and then primed with Stop & Glo® reagent. The plate was read again, this time for Renilla activity and the data saved for subsequent analysis.

2.4.3. Data analysis of luciferase reporter assay

Each sample was transfected in duplicate and the reporter assay performed in duplicate. The mean of the luciferase counts of the transfected duplicates was taken and the same was performed for the Renilla counts. The following calculation was then used to obtain a normalised value:

$$\frac{\text{Mean Luciferase count}}{\text{Mean Renilla count}/1000}$$

The mean of the normalised duplicates performed in the reporter assay was then calculated. To calculate the fold increase, the control sample in each experiment was given a value of 1 and the sample to be analysed was divided by the control value.

2.5. RNA extraction from cells and tissues

RNA was extracted from cell lines and frozen tissue samples using Tri Reagent (Sigma). TRI reagent is an improved version of the single–step total RNA isolation reagent developed by Chomczynski (Chomczynski and Sacchi, 1987). All tubes and instruments used were RNase free. From cell lines, the cells were trypsinised (section 2.3.2), counted, 10^7 cells resuspended in 1 ml of media and centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1ml Tri Reagent. Frozen tissue was ground up using a mortar and pestle under liquid nitrogen, thereafter 100mg powdered tissue was transferred to a fresh RNase free tube and 1ml Tri reagent added. For fresh tissue, 1ml of TRI reagent was added to 100mg tissue.

Samples were homogenised using a mini pellet pestle for 30 seconds, 200µl chloroform (ICN) added and the samples incubated at room temperature for 10 minutes before centrifuging at 13,000 rpm for 15 minutes at 4°C. The upper aqueous layer was removed to a fresh RNase free tube and 500µl isopropanol added. The samples were incubated at room temperature for 10 minutes then centrifuged for 15 minutes at 13,000rpm at 4°C. The supernatant was removed and the RNA pellet resuspended in 1ml 100% ethanol and centrifuged at 7,500 rpm at 4°C. The pellet was washed in 500µl 70% ethanol and allowed to air-dry before being resuspended in 50µl RNA storage buffer (Ambion) or nuclease free water (Promega). RNA concentrations were measured on the GeneQuant spectrophotometer (section 2.10.1).

2.6. Oligo (dT) primed reverse transcription PCR (RT-PCR)

Expression of messenger RNAs in cells and tissues used in this study was determined by RT-PCR using cDNA pools synthesised by oligo (dT) priming of total RNA.

2.6.1. cDNA synthesis

Approximately 1μg of total RNA extracted from cells or tissues (section 2.5) was used in each reaction. This cDNA was then used as a template for the PCR reaction. The RNA was combined with oligo dT as follows:

RNA (1μg) + H_20	11μl
Oligo dT (50pM) (MWG-Biotech)	1μl

Samples were incubated at 70°C for 5 minutes then chilled on ice before addition of:

RNasin (20 units/μl, Promega)	0.5μl
10mM dNTP mix (Promega)	0.4μl
5 x RT Buffer (Bioline)	4μl
H_20	2.85μl
Bioscript Reverse Transcriptase (Bioline)	0.25μl

Samples were incubated at 42°C for 1 hour and then at 72°C for 10 minutes to inactivate the enzyme. The concentration of the cDNA samples were measured on the GeneQuant (section 2.10.1).

2.6.2. Polymerase Chain Reaction

2.6.2.1. Primer design and oligonucleotide synthesis

Oligonucleotides were obtained from published papers or designed using primer design packages (GenejockeyII, primer3). All primers were synthesised by MWG (MWG-Biotech). For the PCR reaction, primers were used at 50pM. Table 2.5 shows sequences and product sizes of the primer pairs used.

Gene Name	5' sequence	3'sequence	Product Size (bp)
c-kit	ATGCGTGTGTCTATGC GTGTGG	GGATTGGCAGCATTAC ATAAGG	275
Oct-4	GAAGCCGACAACAAT GAGAACC	GCTCCAGGTTCTCTTGT CTACC	517
Dazl	ATCCTCCTTATCCAAG TTCACC	ACTGTCTGTATGCTTCG GTCC	261
RBM	GTAATTGCCATAGTCA CAGTATCC	AAGCAGAGCAAGAAGT GGTCC	347
ERβ	CCAATGTGCTAGTGAG CCG	AACTCACGGAACCGTG CCG	393
ERα	TGGTCAGTGCCTTGTT GGATGC	TGTCCAGGAGCAAGTT AGGAGC	290
GFR α-1	TCCTATGAAGAACGA GAGAGGC	AAGCAGTCTTCCAGGT CATTGC	285
Nanog	AGGGTCTGCTACTGAG ATGCTCTG	CAACCACTGGTTTTTCT GCCACCG	363
DAX-1	GTCCAGGCCATCAAG AGTTTC	CAGCTTTGCACAGAGC ATCTC	418
AR	GAGGAACAGCAGCCT TCACAGCAGC	GCTGCTGCTGAAGAAG TTGCAT	266
SGP-1	TAAGGCTAACGAGGA CGTCTGC	GCCTGGACCAGATTCT GCTCAT	484
SGP-2	CATCTGGCATCATAGA CACGCT	ACACAGTGCGGTCATC TTCACC	460
GATA-1	TGTGTGAACTGTGGAG CAACGGC	AAATAGAGGCCGCAGG CATTGCA	362
β-actin	GTGGGCCGCTCTAGGC ACCAA	CTCTTTGATGTCACGCA CGATTTC	540
LDH-C4	CCCTTGTTGACGCTGA TACGA	TTAAGGACCTCATAGC CGCCT	582
GAPDH	CTGCACCACCAACTGC TTAGC	ATGCCAGTGAGCTTCC GTTC	288

Table 2.5 RT-PCR primer sequences

2.6.2.2. PCR reaction

Two different Taq polymerases were used during the course of the studies.

BioTaq (Bioline)

10x PCR buffer (Bioline)	2.5µl
10mM dNTP mix (Promega)	0.5µl
50mM $MgCl_2$ (Bioline)	0.75µl
Forward Primer	0.5µl
Reverse Primer	0.5µl
BioTaq DNA Polymerase (Bioline)	0.25µl
H_20 (Promega)	18 µl
cDNA	1µl
Total Volume	25µl

RedTaq (Sigma)

10x PCR buffer (Sigma)	2.5µl
10mM dNTP mix (Promega)	0.5µl
Forward Primer	0.5µl
Reverse Primer	0.5µl
RedTaq DNA Polymerase (Sigma)	1µl
H_20 (Promega)	19µl
cDNA	1µl
Total Volume	25µl

In general, 35 cycles of amplification were carried out as detailed below:

Initial denaturation	94°C	2 minutes	
Denaturation	94°C	30 seconds	┐ 35 cycles
Specific primer annealing temperature	X°C	30 seconds	┘
Extension	72°C	1 minute / kb of product	
Final extension	72°C	10 minutes	

Products were analysed on a 1% agarose gel (section 2.7).

2.7. Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse double stranded DNA's after PCR amplification or restriction endonuclease digestion. All gels were prepared and run using 1X TAE buffer (section 2.13.4). For DNA sizes below 1kb, a gel prepared with 2% (w/v) agarose (Bioline) was used, for pieces of DNA above 1kb a gel containing 1% w/v agarose was used. Ethidium bromide (Sigma) was added to the gel at a concentration of 0.5μg/μl, ethidium bromide intercalates between DNA bases and fluoresces under UV light and allows visualisation of DNA bands within gels. In order to monitor the migration of DNA during electrophoresis, samples (10-20μl) were mixed with 3μl of Orange G loading dye (section 2.13.4). DNA sizes were detected by loading 5μl of DNA ladder (Hyperladder IV (Bioline) for fragment sizes below 1kb and Hyperladder I (Bioline) for fragments larger than 1kb) into one or more wells within the gel. Gels were run at 10V/cm in a Bio-Rad electrophoresis tank. Following electrophoresis DNA was visualised on a UV transilluminator (GRI Syngene) and photographed using a BioRad camera and integration control unit.

2.8. Cloning

Partial and complete cDNA's generated by PCR or cut and purified from plasmid vectors were cloned into variety of vectors prior to transient transfection.

2.8.1. Cloning of RT-PCR products

PCR products were cloned into TOPO-pCRII vector (Invitrogen) according to manufacturer's instructions. Briefly, 100ng of PCR product was mixed with 1μl salt solution (Invitrogen), 1μl TOPO vector and 2μl sterile H_20. The mixture was incubated at room temperature for 10 minutes before 2μl of the mix was transformed (section 2.8.5) into 50μl XL-1 Blue cells (Stratagene). The entire transformation reaction was spread onto LB Agar plates containing ampicillin at a final concentration of 50μg/ml and incubated inverted overnight at 37°C.

2.8.2. Restriction enzyme digestion of DNA

Restriction enzymes were purchased from New England Biolabs (NEB) or Promega. In general, 1μg of plasmid DNA was mixed with 5μl 10x appropriate enzyme reaction buffer, 5μl 10X BSA (if required), 2.5μl appropriate enzyme(s) and made up to 50μl with sterile H_20. The mix was incubated at 37°C for at least 2 hours. A small aliquot (2-5μl) of the reaction mixture was analysed on an agarose gel to check for complete digestion. To ensure that re-ligation of linearised plasmid vectors did not occur they were incubated with 5 units of shrimp alkaline phosphatase (SAP, Promega) per μg DNA for 15 minutes at 37°C. The sample was then heated to 65°C for 15 minutes to kill any remaining SAP activity. Incubation with SAP catalyses the dephosphorylation of 5' phosphates from DNA preventing recircularisation and religation of the vector.

2.8.3. Extraction of DNA fragments from agarose gels

Following digestion, DNA fragments which were to be cloned into plasmid vectors were separated from vector DNA by gel electrophoresis. The DNA band containing the correctly sized fragment was cut out of the gel using a scalpel and purified using the Qiagen Gel extraction kit. The gel was weighed and 3 volumes of Buffer QG (Qiagen) added and the sample incubated at 50°C for 10 minutes until the gel slice had dissolved. The sample was loaded onto a QIAquick spin column and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded, 750μl of Buffer PE was added and the column left to stand for 3-5 minutes before centrifuging for 1

minute at 13,000 rpm to wash the column. Again, the flow-through was discarded and the sample was eluted with the addition of 50µl 10mM Tris-Cl pH 8.5 to the centre of the column followed by a final spin at 13,000rpm for 1 minute. The sample was analysed using the GeneQuant spectrophotometer (section 2.10.1).

2.8.4. Ligation of DNA inserts into plasmid vectors

After preparation and purification, ligation of the insert into the linearised SAP-treated vector was carried out using T4 DNA ligase (NEB). Generally, the ratio of insert to vector was 3:1 and was calculated using the following equation:

$$\text{Xng insert} = \frac{(\text{Y base pairs of insert})(50\text{ng Vector})}{\text{Base pairs of vector}}$$

The insert and vector were mixed with 1µl of T4 ligase buffer and 1µl T4 DNA ligase in a total volume of 10µl and incubated overnight at 16°C; thereafter 2µl of this mix was used for transform competent *E.coli* (section 2.8.5).

2.8.5. Transformation of competent *E.coli*

Plasmid DNA was propagated in *E.coli* following transformation. This was carried out by adding 2µl (approximately 10ng DNA) of ligation mix (section 2.8.4) to 50µl of subcloning grade XL1-Blue competent cells (Stratagene). The mixture was placed on ice for 30 minutes. The cells were heat shocked by incubating at 42°C for 45 seconds and then returned to ice for 2 minutes. Following this, 950µl of SOC medium (section 2.13.5) was added and the cells incubated at 37°C whilst being shaken at 225 rpm for 1 hour. The bacterial cells were spread onto LB agar plates (section 2.13.5) containing the correct antibiotic using aseptic technique and the plates were incubated inverted overnight at 37°C. Bacterial colonies were isolated and propagated as below (section 2.8.6).

2.8.6. Small scale preparation of plasmid DNA

Single bacterial colonies were picked from LB-agar plates, inoculated to 10mls LB-broth containing antibiotics and incubated overnight in a rotary shaker at 37°C, 225

rpm. The next day, mini-preps were performed using the Promega Wizard ® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions. In brief, 2ml of the overnight culture was centrifuged at 13,000 rpm for 2 minutes. The medium was removed and the pellet resuspended in 250µl cell resuspension solution (50mM Tris-HCL pH 7.5, 10mM EDTA and 100µg/ml RNase); thereafter cells were lysed and denatured by the addition of 250µl cell lysis solution (0.2M NaOH, 1% SDS) and the samples were mixed by inverting 4 times. Alkaline protease solution (10µl) was added to the mixture and the samples inverted a further 4 times before they were left to incubate at room temperature for exactly 5 minutes. The reaction was neutralised with 350µl neutralisation solution (1.32M KOAc, 6.4% glacial acetic acid pH 4.8). Samples were mixed by inverting and centrifuged at 13,000 rpm for 10 minutes in order to pellet cell debris, genomic DNA and proteins. The cleared lysate, which contained the plasmid DNA, was loaded into a spin column and centrifuged for 1 min. The column was washed using 500µl column wash solution (200mM NaCl, 20mM Tris-HCL pH 7.5, EDTA and 47.5% v/v ethanol), centrifuged at 13,000 rpm and the flow-through discarded. This step was repeated using 250µl column wash solution. The column was transferred to a sterile 1.5ml tube, 30µl nuclease free water (Promega) was added and the samples were centrifuged for 2 minutes at 13,000 rpm to elute the plasmid DNA. The concentration and purity of the DNA was measured using the Gene Quant (section 2.10.1). Restriction enzyme digestion (section 2.8.2) followed by agarose gel electrophoresis (section 2.7) was carried out to check that the plasmid contained the insert of the correct size.

2.9. Sequence analysis

Sequence analysis of cloned DNA products was carried out to ensure that the inserts cloned into vectors had no errors in the sequence and that they had been introduced in the correct orientation. Primers used for sequencing are shown in Table 2.6. Primers were diluted to 20µM and 7µl was used for each sequencing reaction. At least 100ng DNA, either plasmid or PCR product was required for sequence analysis. Samples were sequenced by the MRC Human Genetics Unit Automated DNA Sequencing Service using a ABI 377 sequencer. Sequences were analysed using 2

programmes, GeneJockeyII and Vector NTI, and compared to sequences lodged in GenBank using Blast searches.

Name	Primer sequence 5'→ 3'
CMV	CGCAAATGGGTAGGCGTG
T7	TAATACGACTCACTATAGGG
SP6	ATTTAGGTGACACTATAG
ERβ 5'	TCATCCGCGGTCACTGTGACTGGAGGTTCTG
M13 Forward	GTTTTCCCAGTCACGAC
3.0 Reverse	GAGTTAGCTCACTCATTAGGC

Table 2.6. Sequencing primers

2.9.1. Glycerol stocks

Following plasmid preparation, analysis by restriction digestion and sequencing glycerol stocks were prepared so more plasmid could be grown up at a later date. Briefly, 500µl of the bacterial culture was added to 500µl of glycerol (Sigma), vortexed thoroughly and stored at -80°C.

2.10. Caesium chloride preparation of plasmid DNA

For successful transfection, DNA needs to be of high purity therefore large-scale purification of plasmid preparations based on the method established by Ish-Horowicz and Burke, (Ish-Horowicz and Burke, 1981) followed by banding on a CsCl gradient was undertaken as follows: 500ml terrific broth (Mo Bio Laboratories) containing the appropriate antibiotic was inoculated with 1ml mini-prep culture or inoculated from a frozen glycerol stock (see section 2.9.1) and grown up overnight at 37°C (225 rpm). The next day, the culture was split between two 250ml plastic centrifuge tubes (Nalgene) and centrifuged in a J2-21 Beckman centrifuge using a JA-14 rotor at 6000 rpm for 10 minutes at 4°C to pellet the bacteria. The supernatant was carefully resuspended by gentle shaking in 36ml solution P1 (section 2.13.4) followed by 36mls of solution P2 (section 2.13.4) and gently mixed. The solution was incubated for exactly 5 minutes at room temperature because if the cells are left

any longer, they will lyse and release genomic DNA. After this, 36ml chilled solution P3 (section 2.13.4) was added and the contents incubated on ice for 15 minutes; this step precipitated the proteins and high molecular weight DNA. The samples were then centrifuged at 7000 rpm for 10 minutes at 4°C. The supernatant was filtered through miracloth (Calbiochem) to remove any precipitate into clean centrifuge tubes to which 0.7 volumes of isopropanol (ICN) was added and gently mixed. The tubes were centrifuged at 7000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 4ml TE buffer. The sample was extracted with phenol:chloroform (1:1) by vigorous inversion following the addition of 2mls Tris saturated phenol (Sigma) and 2mls chloroform (ICN) and centrifuged at 2500 rpm for 5 minutes. The aqueous layer was removed to a fresh tube, 4 ml chloroform added, the sample again inverted vigorously and the centrifugation step repeated.

The resulting aqueous phase was transferred to a clean tube and the DNA precipitated by addition of 1/10 volume of 3M NaOAc pH 5 and 2 volumes of 100% ethanol, mixed gently and left at –20°C for at least 15 minutes. The sample was centrifuged for 5 minutes at 8000 rpm at 4°C, ethanol removed and the pellet washed with 1ml 70% ethanol and centrifuged again. The supernatant was removed and the pellet allowed to air-dry before being resuspended in 5.6ml TE buffer.

To purify the plasmid DNA, banding on a caesium chloride gradient was performed. The gradient was prepared by adding 1.77g CsCl (Roche) to 1.4ml of the sample plus 40µl ethidium bromide solution (10mg/ml, Sigma). The mixture was heated at 37°C for 5 minutes to dissolve the CsCl before centrifugation at 13,000 rpm for 5 minutes to pellet any debris.

The mixture was added to 2ml polyallomer "Re-Seal Ultra" centrifugation tubes (Kendro Laboratory Products) avoiding any air bubbles, before sealing with a plug and crown (Kendro Laboratory Products). Samples were centrifuged in the Sorvall

Microultracentrifuge using the S150-AT rotor, total centrifugation time was 2.5 hours and the following speeds were used:

Speed (rpm)	Time (minutes)
150,000	80
130,000	25
120,000	10
100,000	10
80,000	25

After ultracentrifugation, two DNA bands were visible; the lower band corresponds to plasmid DNA whilst the upper band represents contaminating genomic DNA; RNA is found in the pellet at the bottom of the tube. The plasmid DNA bands were recovered by holding the tube in a retort stand and carefully inserting an 18-gauge needle and syringe. DNA was drawn out into the syringe and transferred into a fresh 15ml tube (Costar).

To remove the ethidium bromide from the plasmid DNA, an equal volume of CsCl saturated with isopropanol was added and the samples mixed by shaking. When the mixture settled, two phases were visible; the upper aqueous phase, coloured pink, contained the ethidium bromide, and this was removed using a Pasteur pipette. This process was repeated until both phases appeared clear. Subsequently, DNA was precipitated from the lower phase by the addition of 1/10 volume 3M NaAc (Sigma) and 3X volume 70% ethanol, after gentle mixing samples were stored at -20°C for at least 1 hour to allow the DNA to precipitate. Tubes were warmed to room temperature and centrifuged at 7000 rpm for 10 minutes, the supernatant discarded, the pellet washed using 70% ethanol and centrifuged again at 13,000 rpm for 10 minutes. The supernatant was discarded and the pellet allowed to air-dry before DNA was resuspended in 500μl TE buffer.

Any contaminating RNA was removed by RNase treatment: 2μl RNase A (Sigma) was added to the samples and incubated at 37°C for 15 minutes. A final phenol:chloroform extraction was then carried out by addition of 250μl Tris-saturated phenol and 250μl chloroform to the samples, which were then mixed by shaking. Samples were centrifuged at 13,000 rpm for 5 minutes, the upper aqueous layer removed to a fresh tube and 250μl chloroform added, centrifuged again and the aqueous layer removed to a new tube. To this, 1/10 volume 3M NaAc (Sigma) and 3X volume 100% ethanol was added and incubated at -20°C for at least 30 minutes to precipitate the DNA. The sample was then centrifuged for 5 minutes at 13,000 rpm, ethanol removed and the pellet washed with 200μl 70% ethanol and centrifuged again. The ethanol was removed and the pellet allowed to air-dry before being resuspended in 250μl TE buffer. The sample was quantified using the GeneQuant (section 2.10.1) and stored at -80°c.

2.10.1. GeneQuant analysis

Concentration and purity of nucleic acid samples was performed using the GeneQuant spectrophotometer (Pharmacia Biotech). When measuring optical density at 260nM, a reading of 1 is equivalent to 50μg/ml dsDNA or 40μg/ml ssDNA or RNA. The machine was blanked using nuclease free water then 10μl of the sample was placed in the cuvette and analysed. The 260nM:280nM ratio gives an indication of the purity of the sample. A ratio of 1.7 for DNA and 2.0 for RNA is considered to represent a sample free of any degradation or protein contamination.

2.11. RNA interference

2.11.1. Design of siRNAs

Mammalian RNA interference as first described by Elbashir et al (2001), was used in this thesis to enable sequence specific knockdown of target genes. For siRNA studies in mammalian cells, most reports have used a 21mer RNA sequence corresponding to the cDNA of interest (see Chapter 1, section 1.10.1 for details). Many companies offer web-based design tools to aid in siRNA design as well as list of sequences that have been shown to achieve good levels of knockdown. For this study, three web

based design tools were used, Ambion, www.ambion.com, Dharmacon, www.dharmacon.com and Clontech, www.clontech.com. These companies apply certain algorithms to choose a siRNA, which should give good silencing efficiency. These methods are not guaranteed to work so all 3 different design tools were used and a sequence that was chosen by all three was selected, hopefully increasing the chance of obtaining a sequence that will give good silencing efficiency. The siRNAs for ERβ and GFP shown in Table 2.8 were chosen in this manner with the GAPDH sequence either provided as a control sequence, and the others published on the Dharmacon website (www.dharmacon.com).

Targeted Gene	siRNA target sequence
GFP	AAGCTGACCCTGAAGTTCATC
GAPDH	Sequence not given, provided by Ambion
β-tubulin	AAGACAGAGCCAAGUGGACUC
α-tubulin	AAGAUAUUGAGCGUCCAACCU
Lamin A/C	AAGAAGCAGCUUGACGAUGAG
ERβ	AAGAAGAUAAUGGUCAAGCUU

Table 2.7 siRNA target sequences

2.11.2. In vitro transcription of siRNAs

An alternative to using chemically synthesised siRNAs is to construct the siRNA using in vitro translation with T7 polymerase. This is a cheaper but just as effective method to produce siRNAs. An overview of the procedure is shown in Figure 2.2. The Silencer ™ siRNA construction kit (Ambion) was used in this study, according to the manufacturer's instructions. This method was used to construct a GFP siRNA (Table 2.8) and a GAPDH siRNA (control sequence, provided by Ambion).

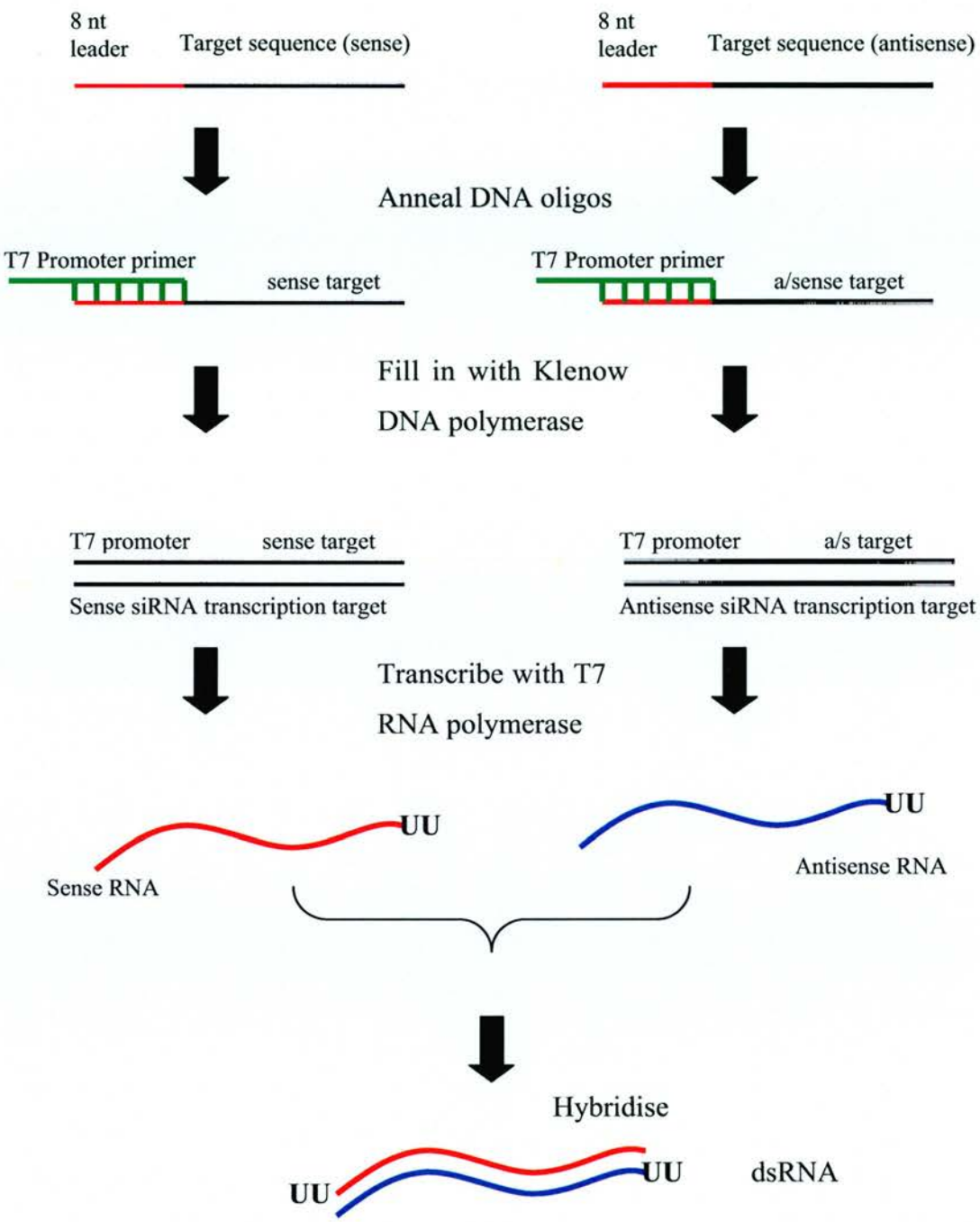


Figure 2.2 In vitro transcription of siRNAs using the Silencer siRNA Construction kit (Ambion).

Two 29-mer DNA oligonucleotides were designed with 21nt encoding the desired siRNA (see section 2.11.1 on designing siRNA) and a 8nt "leader sequence" which is complementary to the T7 promoter primer. Oligonucleotides were synthesised by

MWG. These 2 oligonucleotides were hybridised to a T7 promoter primer in separate reactions. All reagents were obtained from Ambion.

T7 promoter primer	2µl
DNA hybridisation buffer	6µl
Sense or antisense siRNA oligonucleotide template	2µl

The mixture was heated to 70°C for 5 minutes and then incubated at room temperature for 5 minutes. The 3' ends of the hybridised oligos were extended by the Klenow fragment to create double stranded siRNA transcription templates. The following was added to the hybridised oligonucleotides:

10x Klenow reaction buffer	2µl
10x dNTP mix	2µl
Nuclease free water	4µl
Exo-Klenow	2µl

The mixture was gently mixed by pipetting and incubated at 37°C for 30 minutes. The sense and the antisense templates are separately transcribed by T7 RNA polymerase and then the transcripts are hybridised together to create dsRNA which consists of the 5' leader sequence, the 19nt target specific siRNA and a 3' UU. Transcribing the templates separately reduces potential competition between the templates for the transcription reagents, which could limit the synthesis of one of the strands of the siRNA duplex. For each of the templates, the following transcription reactions were set up:

Sense or antisense siRNA hybridised and Klenowed template	2µl
Nuclease free water	4µl
2x NTP mix	10µl
10x T7 reaction buffer	2µl
T7 enzyme mix	2µl

The transcription reactions were incubated for 2 hours at 37°C. The sense and the antisense transcription reactions were combined into a single tube and the incubation was continued overnight.

The dsRNA that was made still contained the single stranded 5' leader sequences that required to be removed. This was done by digesting the dsRNA with RNase and the DNA template was removed by treatment with DNase.

Transcribed dsRNA	20µl
Digestion buffer	6µl
Nuclease free water	48.5µl
RNase	3µl
DNase	2.5µl

The mixture was then incubated for 2 hours at 37°C. The siRNA was purified using the glass fibre filter cartridges provided with the kit. siRNA binding buffer (400µl) was added to the nuclease digestion reaction and incubated for 2-5 minutes at room temperature. siRNA wash buffer (100µl) was added to a filter cartridge and then the siRNA containing the siRNA binding buffer was loaded onto the filter cartridge. The sample was centrifuged at 10,000 rpm for 1 minute and the flow through was discarded. The cartridge was washed with 500µl of siRNA wash buffer and centrifuged at 10,000 rpm for 1 minute, the flow through was discarded and this procedure repeated. The filter cartridge was transferred into a fresh tube and 100µl of nuclease free water that was pre-heated to 75°C was added and incubated for 2 minutes at room temperature. The filter cartridge was spun at 12,000 rpm for 2 minutes and the eluted product was then ready for transfection into cells.

2.11.3. Transfection of siRNAs

2.11.3.1. Oligofectamine

Transfections were carried out according to the manufacturer's instructions. Volumes are given for transfection of a 3.5cm glass bottomed dish, for other culture vessels

the volumes were scaled up or down as appropriate. Cells were plated out 24 hours prior to transfection (section 2.3.3). Serum free transfection medium (section 2.13.2) was used to dilute 3μl oligofectamine reagent to a final volume of 15μl in a sterile microcentrifuge tube. In a separate tube, 200nM siRNA or 200nM siRNA plus 2μg plasmid DNA were diluted in serum free medium to a final volume of 175μl. Both tubes were incubated for 10 minutes at room temperature. After incubation, the diluted oligofectamine reagent was added to the siRNA/DNA, mixed gently and incubated together for 20 minutes at room temperature. The oligofectamine/siRNA/DNA complex was added to the cells, which were already bathed in 800μl of serum free media and returned to the incubator. Four hours post-transfection, 1ml complete media containing 3x normal serum was added and the cells were replaced in the incubator. The cells were assayed for gene activity 24-48 hours post–transfection either by luciferase assay (section 2.4), confocal analysis (section 2.11.4), Q-RT-PCR (section 2.12) or immunohistochemistry (section 2.2.9 /2.2.10).

2.11.4. Analysis of transfected cells by confocal microscopy

Cells transfected with a fluorescent protein (section 2.15.4) or immunostained using fluorescent antibodies (section 2.2.10) were observed on a LSM 510 Laser Scanning confocal microscope (Zeiss) using the Argon 488nm laser to visualise green fluorescence, the HeNe1 543nm laser to visualise red fluorescence and the HeNe2 633nm laser to visualise blue fluorescence. The cells were transfected on glass slides or in 3.5cm glass bottomed dishes as optimum imaging of cells is achieved using glass. The media was removed and replaced with 1ml PBS containing 25mM HEPES (Sigma) and observed on a warm (37°C) stage.

2.11.5. Preparation of plasmid vectors containing shRNA's

The pSilencer 3.0-H1 vector (Ambion) was used to express siRNAs to ERβ and Oct-4. This plasmid encodes a small hairpin RNA (shRNA) under the control of an RNA Polymerase III H1 promoter (Figure 2.3). The H1 promoter provides a high level of constitutive expression across a variety of cell types; shRNA-containing vectors provide longer-term reduction in target gene expression and are easier to work with,

as they are DNA, rather than RNA. The vector was linearised with BamH1 and HindIII to allow directional cloning of the siRNA insert.

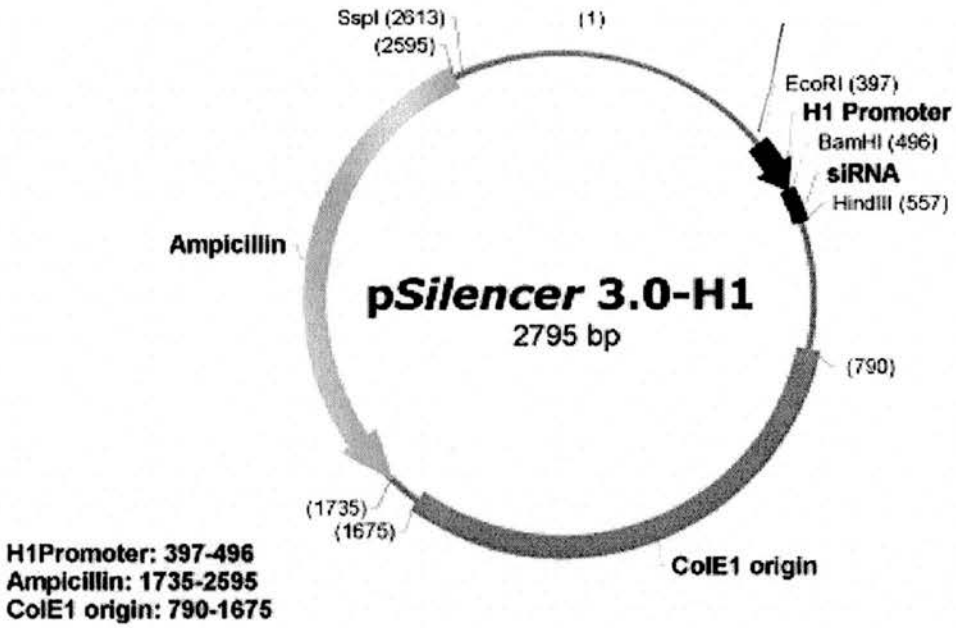
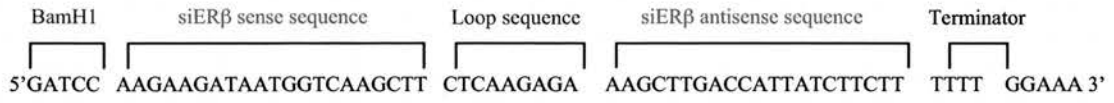


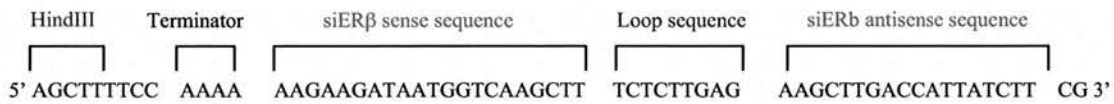
Figure 2.3 pSilencer 3.0 H1 vector (Ambion)

2.11.5.1. Hairpin siRNA Oligonucleotide design

Oligonucleotides (55-60mer) with 3' single stranded overhangs were designed to contain a 19mer hairpin sequence specific to the mRNA target, a loop sequence separating the two complementary domains and a transcription termination sequence. The shRNA oligonucleotides designed for ERβ is shown in Figure 2.4



Sense oligonucleotide



Antisense oligonucleotide

Figure 2.4 ERβ DNA oligonucleotide sequences for hairpin insert and resulting hairpin structure

The oligonucleotides were prepared as a 1μg/μl solution and annealed using the following method:

Sense hairpin siRNA oligo	2μl
Antisense hairpin siRNA oligo	2μl
1 X DNA annealing solution (Ambion)	46μl

The mixture was heated to 90°C for 3 minutes, cooled to 37°C and incubated for 1 hour.

2.11.5.2. Ligation of hairpin siRNA inserts into pSilencer vector

The hairpin siRNA (5µl) was added to 45µl nuclease free water (Ambion) to give a final concentration of 10ng/µl and a ligation reaction was set up as follows:

Diluted annealed siRNA insert	1µl
Nuclease free water (Ambion)	6µl
pSilencer vector (0.5µg/µl)	1µl
10 x T4 DNA ligase buffer (NEB)	1µl
T4 DNA ligase (5 U/µl)	1µl

The ligation reaction was incubated overnight at 16°C, a negative control (no insert) reaction was prepared in parallel.

2.11.5.3. Transformation and identification of hairpin siRNA containing clones

The ligation mixture (10µl) was transformed into XL-1 Blue competent cells (Stratagene) as in section 2.8.5 and propagated onto LB-agar-ampicillin plates at 37°C overnight. Colonies were picked, grown up in medium and plasmid minipreps prepared (section 2.8.6). The plasmids were sequenced (section 2.9) to confirm the presence of the correct siRNA insert. Large-scale plasmid preparations were prepared and purified using CsCl banding (section 2.10) before they were used for cell transfections.

2.12. Quantitative RT-PCR

Quantitative RT-PCR was carried out using the TaqMan analysis system devised by Applied Biosystems. Primers and probes were designed to each DNA target using the Primer Express software package (2.12.2) and where available Assay On Demand™ Gene Expression-Assays (Applied Biosystems) (section 2.12.4) containing pre-validated primer probe sets were used in the study.

2.12.1. Principles of the TaqMan reaction

Sequence specific probes located between the forward and reverse primers were chosen for each sequence. Probes have a reporter dye and quencher attached to the 5' and 3' ends respectively. In this study, the 5' end of the probe was 6-carboxyfluorescein, (FAM) dye labelled, which is the reporter dye and the 3' end is labelled with a 6-carboxy-tetramethyl-rhodamine (TAMRA) quencher dye. Whilst intact, the close proximity of the reporter to the quencher suppresses fluorescence of the reporter (Figure 2.5).

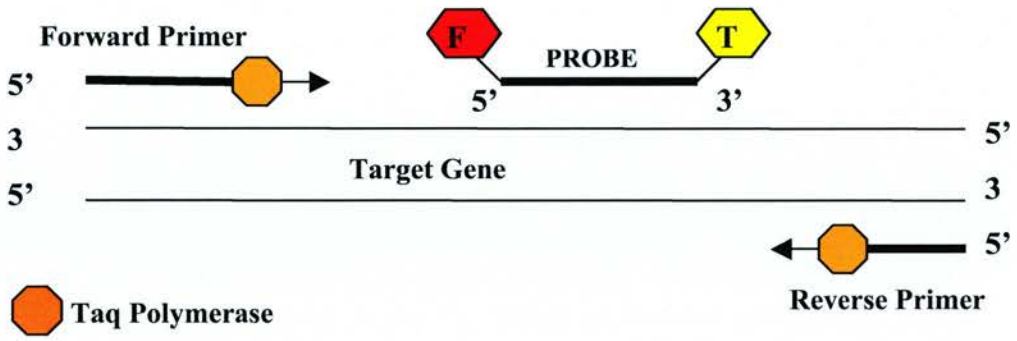


Figure 2.5 Reporter-quencher proximity results in suppression of fluorescence

If the target sequence is present in the sample being amplified, the probe anneals to the target gene cDNA between the forward and reverse primers. The TaqMan probe has a melting temperature 10°C higher than that of the primers and the sequence of either strand can be complementary to the probe. Taq polymerase has nuclease activity in the 5' to 3' direction, cleaving the probe releasing the FAM dye so it is no longer quenched by the TAMRA dye (Figure 2.6). This only takes place if the probe has hybridised to the target sequence of cDNA. The fluorescent activity increases with an increase in the amount of probe cleavage, which in turn is proportional to the amount of PCR product formed. The accumulation of PCR product is measured quantitatively by the sequence detection system.

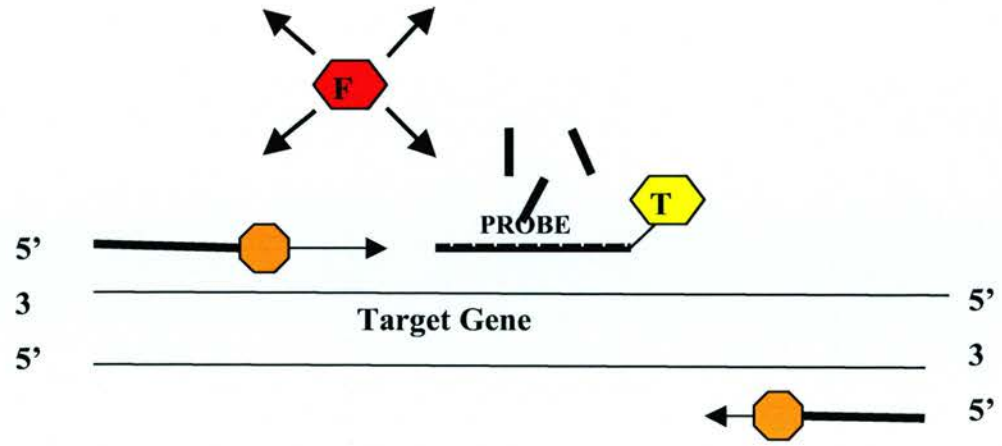


Figure 2.6 Cleavage of the reporter from the quencher

2.12.2. Taqman primers and probes

Table 2.9 shows the sequences of the primer pairs and probes used in this study. Primers were picked from the relevant sequences using PrimerExpress software and purchased from Biosource.

Gene	Forward Primer 5'→3'	Reverse Primer 5'→3'	Probe 5'FAM→3'TAMRA
ERβ	GAAGGCCATGA TTCTCCTCAAC	TCCGGCTACTC TCTGCTTCCT	CCAGTATGTACCCC TTGGCTACCGCAA
AR	TTTGCACCTGACTT GGTTTTCA	CATCCTCACACACTG GCTGTACA	TGAGTACCGCATGC ACAAGTCTCGG

Table 2.8 Taqman primer/probe sequences

2.12.3. Optimisation of primers and probe concentration

For efficient amplification, the optimal concentration for the primer pair and the probe needs to be determined.

2.12.3.1. Primer optimisation

The combinations of forward and reverse primers shown in Table 2.10 were set up in a reaction in triplicate; the probe concentration used was 100nM.

50 nM Forward 50 nM Reverse	50 nM Forward 300 nM Reverse	50 nM Forward 900 nM Reverse
300 nM Forward 50 nM Reverse	300 nM Forward 300 nM Reverse	300 nM Forward 900 nM Reverse
900 nM Forward 50 nM Reverse	900 nM Forward 300 nM Reverse	900 nM Forward 900 nM Reverse

Table 2.9 Primer Optimisation Matrix

The average ΔCt values for ERβ and AR were evaluated by subtracting the internal control (18S) Ct value from the experimental sample Ct value (section 2.12.7). The best primer pair combination is the one that gives the minimum ΔCt with the lowest concentration. In the case of ERβ and AR, this was found to be 300 nM forward and 300nM reverse and this was used throughout.

2.12.3.2. Probe optimisation

The amount of probe used also must be optimised to prevent probe-limiting fluorescence. A reaction was carried out using the optimised primer pair combination of 300nM forward primer/300nM reverse primer with increasing probe concentration from 50nM to 200nM. The sample analysed was mouse ovary RNA in the case of ERβ (shown in Figure 2.7) and mouse testis RNA in the case of AR. The lowest ERβ - 18S Ct value without using excess probe was chosen. In the case of both ERβ and AR, this was found to be 100nM.

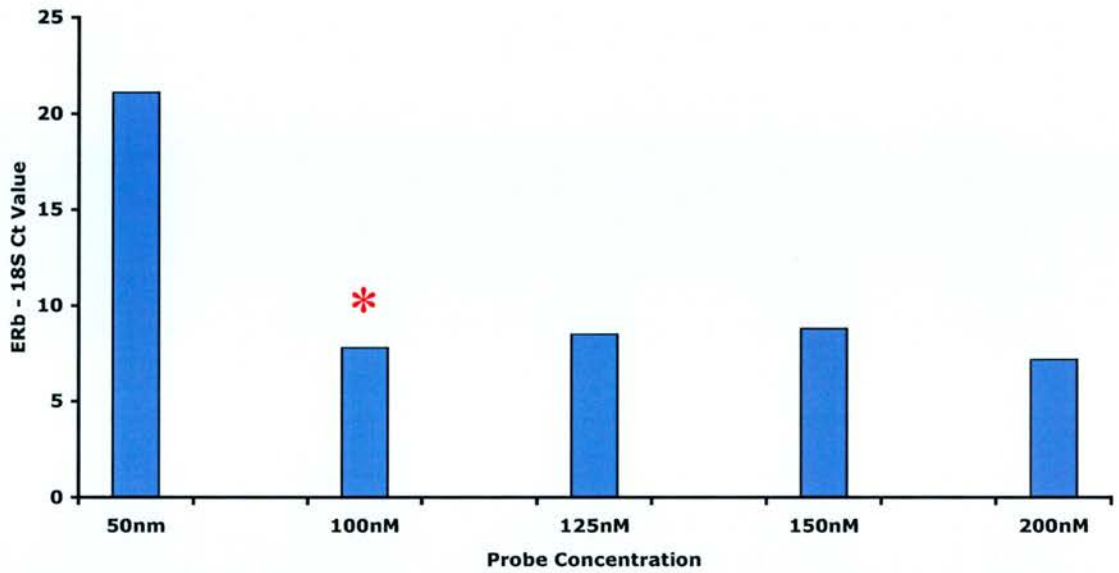


Figure 2.7 ERβ probe optimisation

2.12.4. Assay-On-Demand™

The Assay-On-Demand™ Gene Expression system (Applied Biosystems) are optimised, validated ready-to-use primer/probe assays for use with the TaqMan system. In this study, Assay-On Demand systems were used for c-kit (Cat no. Mm00445241_m1) and Oct-4 (Cat no. Mm00658129_gH).

2.12.5. Preparation of cDNA pools for TaqMan analysis

Using Applied Biosystems' TaqMan Reverse Transcription Reagents kit, cDNA was synthesised using random hexamers according to the following reaction

Nuclease Free water	1.85µl
10x RT Buffer	1.0µl
25mM $MgCl_2$	2.2µl
dNTP's	2.0µl
Random Hexamers	0.5µl
RNase Inhibitor	0.2µl
MultiScribe RT	0.25µl
RNA (100ng/µl)	2.0µl

The reagents were added together in a sterile 0.2ml thin walled PCR tube (CLP) and placed in a thermocycler programmed with the following cycle times:

25°C	20 minutes
42°C	60 minutes
95°C	5 minutes

2.12.6. Q-RT-PCR reaction

Primers were diluted to 5µM and probes to 5µM in TE buffer (10mM Tris; 1mM EDTA). Samples to be tested were run in triplicate on a 96 well MicroAmp optical reaction plate (Applied Biosystems). For each sample, a tube containing 37.5µl 2x universal PCR master mix (Applied Biosystems), 1.65µl each primer, 1.5µl probe, 1.125µl 18S primer/probe mix, and 24.075µl H_20 was prepared and 7.5µl of cDNA (section 2.12.5) added to give a final volume of 75µl; 25µl of this mixture was added to each of three wells in the plate. Appropriate positive and negative controls were included on each plate.

For the Assay on Demand samples, 37.5µl 2x PCR master mix (Applied Biosystems), 3.75µl Assay on Demand primer/probe mix, 1.125µl 18S primer/probe mix and 25.125µl H_20 were combined together, 7.5µl cDNA added and 25µl of the mixture added per well. The plate was sealed with an ABI prism optical adhesive optical cover (Applied Biosystems) and loaded onto the ABI 7900 sequence detection system.

2.12.7. Analysis of results – comparative Ct method

Taqman PCR results are shown as an amplification plot. This plot gives the amounts of the reporter dye generated during amplification and is directly related to the formation of PCR products. An example of an amplification plot is shown in Figure 2.8. The FAM Ct value obtained corresponds to the cycle number at which the fluorescence due to amplification of the PCR product reaches a significant level above the background fluorescence (threshold level). The threshold level is determined as the point when an increase in signal is associated with an exponential increase of PCR product.

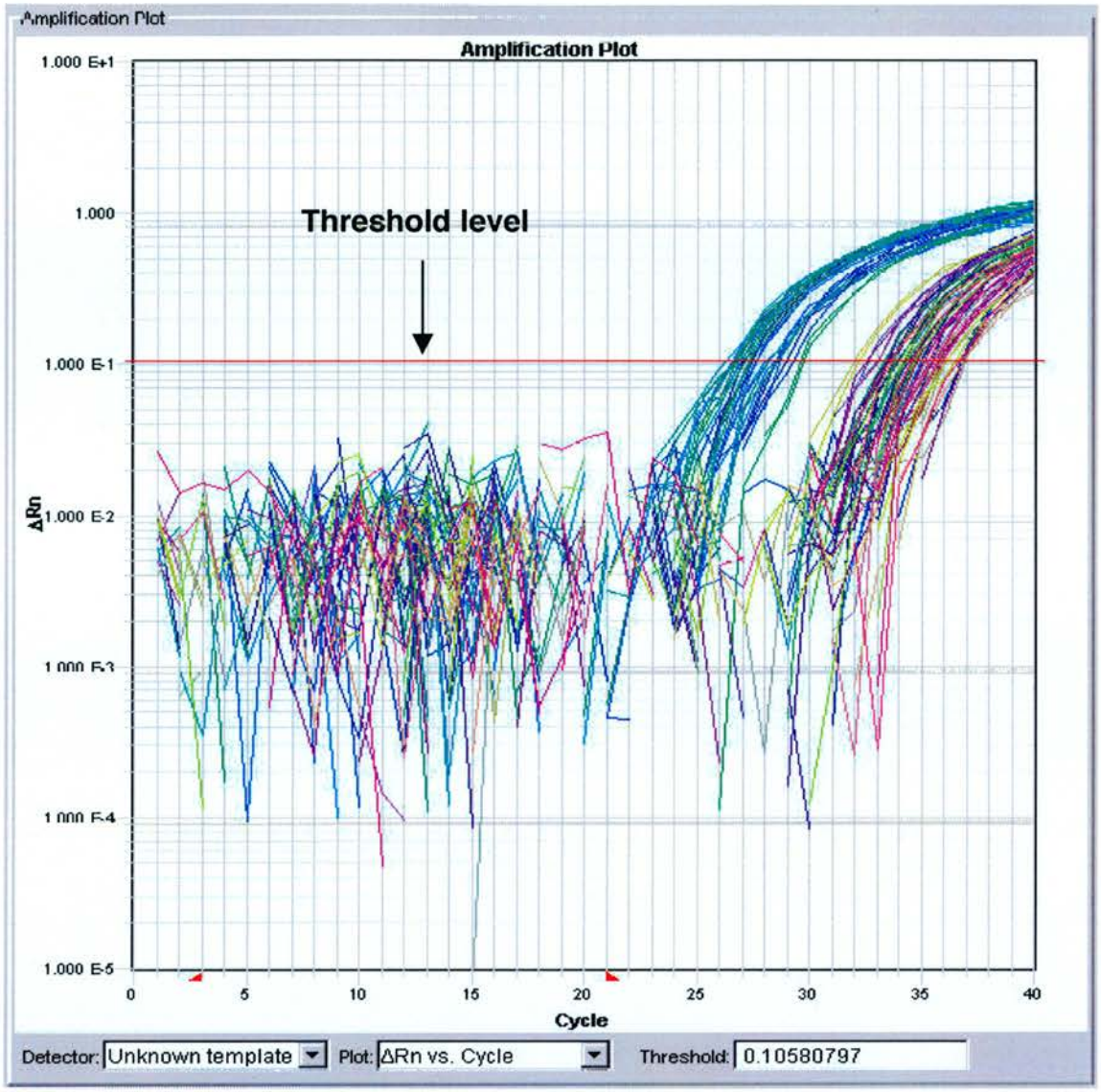


Figure 2.8 Example of an amplification plot after a successful Taqman RT-PCR reaction.

The Ct value is directly related to the amount of PCR product and therefore is related to the original amount of target mRNA present in the reaction. A change in Ct value of one equates to a two-fold difference in initial template concentration, for example, if the Ct value decreases by one compared to the control sample, this is due to a doubling of the initial template concentration. The 18S ribosomal RNA control is a measure of the mRNA content of the sample and is used as an internal control for mRNA variation between samples. To analyse the results, the ΔCt value was

calculated where ΔCt is the difference between the FAM Ct and the 18S Ct value and enables the amplified signal to be normalised against the total mRNA content. The mean ΔCt between triplicates on the plate was calculated. This was used to calculate the ΔΔCt, which is the difference between the ΔCt of a treated sample compared to the control sample in that experiment. Within each experiment the ΔCt is related to its own control. The amount of amplified target is given the value of $2^{-\Delta\Delta Ct}$. This function is based on the mathematical equation that describes the exponential amplification of the PCR reaction $X_n = X_o \times (1+ E_x)^n$ where X_n is the number of target molecules at the threshold at cycle n, X_o is the initial number of target molecules, $(1+ E_x)$ being the efficiency of the target gene amplification and n equalling the number of cycles. Assuming that the efficiencies of the target and internal control reactions are equal (section 2.12.8), the $2^{-\Delta\Delta Ct}$ value is a measure of relative quantification and in this study is used to show the fold increase or decrease in mRNA expression of the samples relative to the control with each control always having a $2^{-\Delta\Delta Ct}$ value of 1.

2.12.8. Validation

To analyse the results using the comparative Ct method, a validation experiment must be carried out to demonstrate the efficiencies of target and endogenous control amplifications are approximately equal. Mouse ovary cDNA was diluted from 1ng down to 0.01ng and a reaction performed. The average ΔCt value was calculated (section 2.12.7) and was plotted against the log amount of input RNA. The absolute value of the slope of log input RNA versus ΔCt should be <0.1. Relative efficiency of ERβ target and 18S reference is shown in Figure 2.9. The slope in this case is 0.0741, which passes the test. The advantage of using the comparative Ct method is that the need for a standard curve to be run on each plate is eliminated.

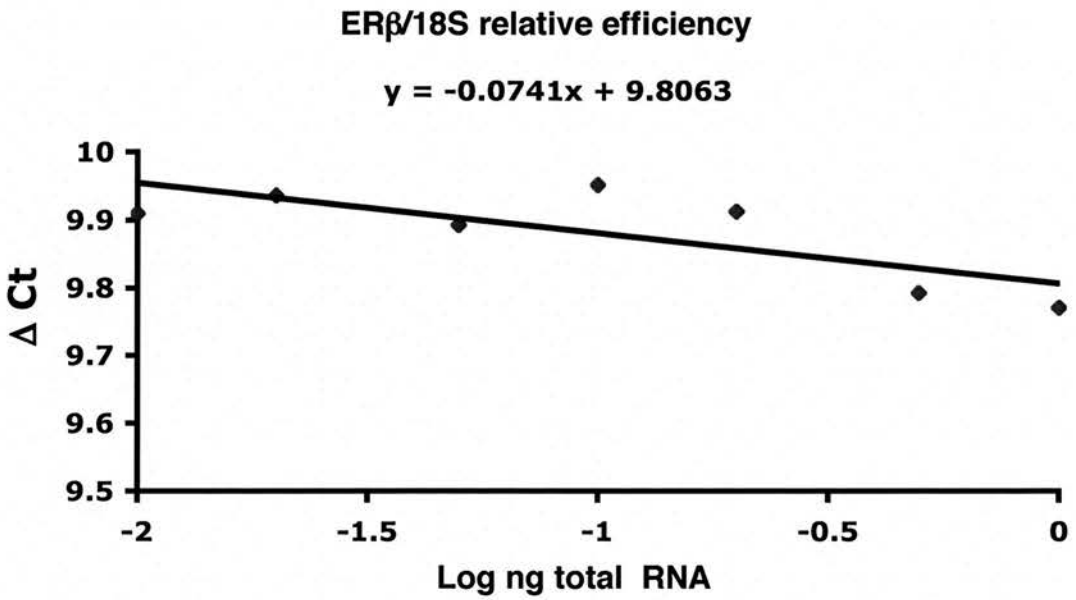


Figure 2.9 Plot of log input RNA amount versus ΔCt

2.13. Statistical analysis

Where required, data was analysed for statistical significance using the GraphPad Prism software package. Statistical significance was determined by either using a paired students t-test or by one-way analysis of variance (ANOVA). Results were deemed significant when $P < 0.05$.

2.14. Commonly used solutions

2.14.1. Immunohistochemistry

Modified Davidson's Fixative	37-40% formaldehyde (Sigma)	30%	final
	Ethanol (Haymans)	15%	final
	Glacial acetic Acid (BDH)	5%	final
	Water	50%	final

TBS	Tris (Sigma)	60.5g
	NaCl (Sigma)	87.6g
	HCl (BDH)	300ml
	Adjust pH to 7.4 using concentrated HCl	

2.14.2. Tissue culture

Complete medium

Dulbecco's Modified Eagles Medium (DMEM, Sigma), supplemented with:
10% Charcoal stripped foetal calf serum (FCS, Gibco)
1% Non-essential amino acids (Sigma)
1% D-(+)-Glucose (45% solution, Sigma)
1% 2mM L-Glutamine (Sigma)
1% Penicillin-streptomycin (Gibco)
1% Fungizone (Gibco)

For studies involving the investigation of steroid hormone receptors, the cells were changed to complete medium made with DMEM containing no phenol-red (Sigma).

Transfection medium

Phenol red free DMEM (Sigma), supplemented with:
1% Non-essential amino acids (Sigma)
1% D-(+)-Glucose (45% solution, Sigma)
1% 2mM L-Glutamine (Sigma)

Freezing Medium

40% complete medium (Section 2.13.2)
50% FCS (GIBCO)
10% DMSO (Sigma)
Filter sterilised before use

2.14.3. Stem cell isolation and sorting

Supplemented EBSS

100mls Earle's Balanced Salt Solution (Gibco)
500µl 100mM sodium pyruvate (Sigma)
112µl sodium lactate (60% w/w) (Sigma)
1ml 200nM glutamine (Gibco)

2.14.4. Molecular biology

0.5M EDTA

Dissolve 186.1g of EDTA (Sigma) in 800ml of dH_20
Adjust to pH 8.0 with NaOH (Sigma)
Make up to 1 litre with dH_20

1 x TE

5ml 1M Tris (Sigma)
1ml 0.5M EDTA (Sigma)
Make up to 500ml with dH_20

3M Sodium Acetate

Dissolve 408.1g $NaAc_3H_20$ (Sigma) in 800ml dH_20
Adjust to pH5.2 with acetic acid (BDH)
Make up to 1 litre with dH_20

50 x TAE

242g Tris (Sigma)
100ml 0.5M EDTA (Sigma)
57.1ml acetic acid (BDH)
Make up to 1 litre with dH_20, use at 1x.

Orange G loading dye

50% 1x TAE
20% Glycerol (Sigma)
0.25% Orange G (Sigma)

Solution P1

50mM Glucose (Sigma)
25mM Tris HCl pH 8 (Sigma)
10mM EDTA (Sigma)

Solution P2

0.2M NaOH (Sigma)
1% SDS (Promega)

Solution P3

3M KAc pH 4.8 (ICN)

2.14.5. Microbiology

LB Broth

Dissolve 25 pellets of LB-Broth (Q-Biogene) in 1 litre of dH_20 and autoclave.

LB Agar

Dissolve 31 pellets of LB-Agar (Q-Biogene) in 1 litre of dH20 and autoclave.

SOC media

20g bacto-trypytone
5g bacto-yeast extract
0.5g NaCl (Sigma)
10ml filter sterilised 2M glucose (Sigma)
Make up to 1 litre with H_20

Ampicillin stock solution

50mg/ml ampicillin (Sigma) in dH_20
Working concentration 50μg/ml.

Kanamycin stock solution

30mg/ml kanamycin (Sigma) in dH_20
Working concentration 30μg /ml

Chapter 3

Steroid receptor expression and modulation in Sertoli cells

3.1. Introduction

The testis has two major functions, the synthesis of steroid hormones and the production of spermatozoa. The adult testis contains three main somatic cell types, namely Sertoli cells, Leydig cells and peritubular myoid cells as well as germ cells at all stages of maturation. Interactions between these cells, some of which involve steroid hormones are responsible for the maintenance of spermatogenesis and fertility.

3.1.1. Sertoli cell development and function

The Sertoli cell is the only somatic cell in direct contact with the germ cells in the seminiferous epithelium. They are the first cells to differentiate within the male fetal gonad and they prevent the germ cells from premature entry into meiosis (Mackay, 2000). The Sertoli cells are the only type which express Sry and they therefore play a role in sex determination and influence the determination of other cell types in the testis (Swain et al., 1998). Sertoli cells first appear in the human fetal testis at approximately 8 weeks of age and by birth, the human testis contains approximately 10% of the adult complement of 4000 million cells (Pelliniemi et al., 1993). In mice, Sertoli cells are thought to be present in the gonad by embryonic day 11.5, the evidence for this comes from the fact that anti mullerian hormone (AMH), which is a Sertoli cell specific gene product during fetal life is first detected in the gonad at this time (Byskov, 1986; Karl and Capel, 1998).

3.1.1.1. Sertoli cell maturation

At puberty, the role of the Sertoli cell alters and the cells become essential for supporting the process of spermatogenesis by interactions with other cells via cell-to-cell contact or by paracrine and endocrine signals (Sharpe, 1994). Sertoli cell differentiation is marked by the development of a tripartite nucleus with a prominent nucleolus (Sharpe et al., 2003). As only immature Sertoli cells can proliferate, the

total Sertoli cell number is determined prior to adulthood. In rodents and in humans, proliferation of Sertoli cells begins during fetal life. In rats and mice, this proliferation declines steadily after birth. Puberty in the male rat is usually recognised to be between 25 and 30 days of age and Sertoli cell proliferation ceases around day 15 (Sharpe, 1994). In the mouse, the proliferative activity decreases after birth and by day 11 the Sertoli cells show their typical adult appearance. The murine Sertoli cell population reaches the level of approximately 18 x 10^5 cells per testis by around day 17 (Vergouwen et al., 1991). Similar findings were reported by Kluin et al. (1984) who found that Sertoli cell proliferation ceased at day 12 (Kluin et al., 1984). In humans and primates Sertoli cell proliferation also occurs in the neonatal period, with in some cases a further phase at puberty (Sharpe, 1994). For example, in the marmoset, it has been shown that adult numbers of Sertoli cells are reached by the end of the neonatal period whereas the rhesus monkey shows the greatest rate of proliferation in the peripubertal period (Plant et al., 1978; Sharpe, 1994; Sharpe et al., 2003).

3.1.1.2. Regulation of Sertoli cell function

Sertoli cell function is regulated by hormones, growth factors and other paracrine factors (Sharpe et al., 2003). Androgens are synthesised from cholesterol in the Leydig cells of the testis resulting in the production of androstenediol and testosterone. Testosterone produced from this conversion can pass into the Sertoli cells where it can be further converted into its more active metabolite dihydrotestosterone (DHT) by the enzyme 5α reductase (Miller, 1988). Oestrogens are synthesised from androgens by the cytochrome P450 aromatase complex (Carreau et al., 1999; Carreau et al., 2003; Hess, 2003; Hess et al., 2001). P450 aromatase converts androstenedione to oestrone and testosterone to 17β-oestradiol (Murata et al., 2002). In rats, aromatase is expressed in fetal and neonatal Sertoli cells but its expression is downregulated during Sertoli cell maturation (Palmero et al., 1995). Aromatase is also highly expressed in the Leydig cells of the testis as well as in some germ cells in mice, rats and the marmoset monkey (Nitta et al., 1993; Rommerts et al., 1982; Turner et al., 2002). Humans express the aromatase enzyme in germ cells and in spermatozoa (Carreau et al., 2003).

FSH is one of the most important regulatory signals involved in the regulation of Sertoli cell function. The first evidence came from experiments where FSH was administered to hypophysectomised rats; this resulted in the maintenance of spermatogenesis in these animals (Greep and Fevold, 1937). FSH has been shown to control the proliferation of Sertoli cells during fetal and early postnatal life and their final differentiation during puberty (Griswold, 1993). The presence of the FSH receptor on Sertoli cells is critical for the hormonal response of the testis to FSH (Heckert and Griswold, 1991). The importance of FSH to Sertoli cell function was further demonstrated by the generation of knockout mice for FSH and the FSH receptor, both of these mice show reduced Sertoli cell numbers in adulthood. In the FSH receptor KO mouse, Sertoli cell numbers are normal at birth but are significantly decreased by day 20 (Abel et al., 2000; Dierich et al., 1998; Kumar et al., 1997). These knockouts are described in more detail in chapter one, section 1.9.5. The ability of FSH to regulate Sertoli cell number can also be shown by experiments in which high levels of oestrogens administered neonatally suppressed FSH resulting in a reduction of Sertoli cell numbers, indicating an indirect impact on testicular function resulting in abnormal spermatogenesis (Arai et al., 1983; Atanassova et al., 1999; Sharpe et al., 1998).

In addition to FSH, androgens are involved in the regulation of spermatogenesis. The study by Johnson et al. (2004), investigated the role of FSH and androgens in relation to Sertoli cell maturation and function in the FSHKO and Tfm mice. They concluded that in the knockouts studied, both FSH and androgens were required to produce the normal number of Sertoli cells in the adult mouse testis but it is androgens that are essential during the neonatal period for normal Sertoli cell function (Johnston et al., 2004). Androgens have also been shown to affect Sertoli cells in culture (Sar et al., 1993).

3.1.2. Steroid hormone receptors

Steroids act on target tissues via specific receptors, which are members of a superfamily of ligand activated transcription factors. Receptors for androgens and

oestrogens are expressed in the male reproductive tract of many species including mouse (Zhou et al., 2002), rat, (Bremner et al., 1994; Fisher et al., 1997; Hess et al., 1997; Sar et al., 1990; Saunders et al., 1998), rabbit (Danzo and Eller, 1979), monkey (Fisher et al., 1997; McKinnell et al., 2001; Saunders et al., 2001), cats and dogs (Nie et al., 2002) and humans (Saunders et al., 2001; Saunders et al., 2002; Suarez-Quian et al., 1999). In the mouse, immunohistochemistry has shown that AR and ERβ are present in Sertoli cells but ERα is absent (Atanassova et al., 2000; Walther et al., 1996; Zhou et al., 2002). The distribution of AR, ERα and ERβ is not uniform throughout different species.

In the adult mouse testis, ERα immunoexpression has been detected in the Leydig cells and the peritubular myoid cells but not in the Sertoli and germ cells (Zhou et al., 2002). Species differences have been reported, for example, Saunders et al. (2001) did not detect ERα immunoexpression in any cell type of the testes in humans, marmosets or macaques (Saunders et al., 2001) although, Fisher et al. (1997) detected ERα in occasional Leydig cells in the marmoset (Fisher et al., 1997). ERα has also been detected in the Leydig cells of cats and dogs (Nie et al., 2002). In comparison, ERβ is found in multiple cell types within the mouse and rat (Saunders et al., 1998; Saunders et al., 2001; Zhou et al., 2002). In the human, ERβ has been immunolocalised to Sertoli cell nuclei and in some, but not all germ cells as well as in Leydig cells and peritubular myoid cells (Saunders et al., 2001). In primate models, ERβ was found in all the cell types of the testis (Saunders et al., 2001).

In rodents and humans, Sertoli cell expression of AR is switched on postnatally. In adults, Sertoli cell expression of AR is stage dependent (Bremner et al., 1994; Suarez-Quian et al., 1999). As well as the Sertoli cells, AR is also found in the other somatic cells of the testis, the Leydig and peritubular myoid cells. AR activity is regulated by the steroid hormone testosterone and its derivative DHT. The androgen receptor plays a role in the feedback regulation of testosterone levels through autocrine feedback on the Leydig cells (Holdcraft and Braun, 2004). AR is not detected in the germ cells of the mouse and rat testis (Bremner et al., 1994; Zhou et

al., 2002) suggesting that the actions of androgens on the germ cells are mediated by the Sertoli cells. A few reports however, claim to have found AR positive germ cells in other species including the rat (Kimura et al., 1993; Vornberger et al., 1994).

3.1.3. Sertoli cells *in vitro*

The culture of Sertoli cells *in vitro* is an important tool for the study of Sertoli cell function. As Sertoli cells are only a small proportion of the adult testis, isolated Sertoli cells are commonly used for functional studies. However most primary cultures of Sertoli cells are established using cells from prepubertal rodents and in most cases, these cells dedifferentiate and fail to proliferate (Karl and Griswold, 1990). Primary Sertoli cell cultures are known to lose their characteristic morphology after enzymatic digestion and become rounded (Bellve et al., 1977). In addition to this, primary Sertoli cells, lose receptor expression and consequently become unresponsive to hormones after 6 to 10 days in culture (Karl and Griswold, 1990).

Several attempts have been made to establish long-term cultures of Sertoli cells. Examples of these include the TM4 (Mather, 1980) and MSC-1 (Peschon et al., 1992) cell lines. However, the TM4 cell line have become dedifferentiated and the MSC-1 cell line has lost FSH receptor expression (Mather, 1980; Peschon et al., 1992). As well as these, two cell lines have been immortalised using SV40 large T antigen. The Sertoli cell line chosen for use in this study was the SK11 cell line (Walther et al., 1996). This line was derived from Sertoli cells of a 10 day old *H-2K^b-tsA58* transgenic mouse (Jat et al., 1991). The mice carry the temperature inducible SV40 large–T antigen which is a conditional immortalising gene under the control of the mouse major histocompatibility complex-1 promoter H-2k^b which is active at various levels throughout various tissues. The tsA58 TAg gene product is functional at the permissive temperature of 33°C but is degraded at the non-permissive temperature of 39°C. The presence of the TAg gene results in the generation of continuously proliferating cells, capable of differentiation following culture at the non-permissive temperature. SK11 cells were established using this method. Primary Sertoli cells were isolated from these mice and cultured in the

presence of interferon-γ, which induces the expression of the SV40 large T antigen, stimulating permanent growth. The cells were continuously passaged until a homogeneous population was obtained. Upon transfer to the non–permissive temperature of 39°C, the temperature sensitive SV40 antigen is inactivated, resulting in differentiation of the cells and a significant reduction in growth (Walther et al., 1996; Walther et al., 1997). After two days of culture at 39°C the cells stopped growing and began to show a morphological change and become flattened. SK11 cells were reported to express mRNAs for AR and FSHR, as well as other Sertoli cell products including α-inhibin, GATA-1, SGP-2, SCF and SF-1 (Walther et al., 1996). Recently, another cell line has been described which conditionally immortalised freshly isolated Sertoli cells again using the SV40 Large T antigen under the control of an ecdysone inducible promoter. These cells expressed SCF, GATA-1 and SGP-2 and were shown to be able to support the survival of germ cells in culture (Hofmann et al., 2003).

3.1.4. RNA interference

As described in Chapter One, RNA interference is a technique which results in sequence specific gene silencing (Elbashir et al., 2001). The development of vector-based delivery systems has allowed more stable expression of siRNAs. When placed in a vector, siRNAs are expressed as fold-back hairpin–loop structures that give rise to siRNAs after intracellular processing (Tuschl, 2002). Transfection of plasmid DNA, rather than synthetic siRNAs, is advantageous in many ways, overcoming problems with RNase contamination and the costs of chemically synthesized siRNAs or siRNA transcription kits. By using a vector based delivery system, longer-term knockdown can also be achieved.

3.1.5. Aims

The studies described in this chapter were designed to determine the impact of steroid hormones (oestradiol and testosterone) on Sertoli cell function and to attempt to knockdown the expression of ERβ using siRNA. All studies utilised the immortalised Sertoli cell line SK11. A cell line was chosen to perform the studies in this chapter due to the drawbacks of using freshly isolated Sertoli cells, which

include morphological changes and the loss of receptor expression. The advantage of using this cell line is that it can be maintained in both an undifferentiated and differentiated state. Initial studies characterised the pattern of gene expression in the SK11 cells and the expression of steroid hormone receptors. Steroid responsiveness was then investigated using transient transfection studies.

The knockdown of ERβ was attempted in the SK11 cell line by the use of RNA interference technology. siRNA methodologies were optimised using the SK11 cells and the expression of ERβ was knocked down in these cells with the use of a short-hairpin siRNA.

3.2. Materials and methods

3.2.1. SK11 cell culture

The SK11 cell line was maintained at 33°C in 5% (v/v) CO_2 in complete media (sections 2.3.1 and 2.13.2). Passaging of the cells was performed every 3-4 days by trypsinisation (section 2.3.2). The cells were induced to differentiate by incubation at 39°C for at least 48 hours before any experimental procedure was carried out.

3.2.2. Immunohistochemistry

Immunohistochemistry was carried out on SK11 cells and on sections prepared from the testis of 10 day old and adult mice using standard methods as detailed in chapter 2, section 2.2.9. The SK11 cells were grown on 2 well or 8 well chamber-well slides (Nalgene Nunc) and immunohistochemistry carried out when the cells reached 60-70% confluency. Antibodies used for immunohistochemistry are shown in Table 3.1.

Antigen	Dilution	Species raised in	Source
ERβ	1:1500	Sheep	Raised in house, (Saunders et al., 2000).
ERα	1:20	Mouse	Novocastra
AR	1:200	Rabbit	Santa Cruz
GATA-1	1:100	Goat	Santa Cruz

Table 3.1 Antibodies used for immunohistochemistry of cultured SK11 cells and testis tissue sections

3.2.2.1. Fluorescent immunohistochemistry

SK11 cells (differentiated and undifferentiated) were plated onto glass 2-well chamberwell slides and left to attach overnight. Immunohistochemistry was performed as in section 2.2.10 using primary and secondary antibodies shown in Table 3.2. The cells were counterstained using To-Pro3 (Molecular Probes) as described in section 2.2.10 and observed using the confocal microscope (section 2.11.4).

	Antigen	Dilution	Species raised in	Source	Dilution
Primary Antibody	β Actin	1:200	Mouse	Sigma	1:200
Secondary antibody	Goat Anti Mouse	1:200	Goat	Molecular probes	1:200

Table 3.2 Primary and secondary antibodies used for fluorescent immunohistochemistry

3.2.3. RT-PCR

Total RNA was extracted from the SK11 cells and from mouse testes (adult and 10 day old) as described in section 2.5. Oligo dT primed cDNA was synthesised, using standard conditions (section 2.6) and a standard PCR reaction was carried out with 30 cycles of amplification (section 2.6.2.2) and a reaction mix containing BioTaq (Bioline). Primer pairs designed to amplify mouse ERα, ERβ and selected Sertoli cell products are given in Table 3.3.

Gene Name	5' sequence	3'sequence	Product size (bp)
ERβ	CCAATGTGCTAGTGAG CCG	AACTCACGGAACCGTG CCG	393
ERα	TGGTCAGTGCCTTGTT GGATGC	TGTCCAGGAGCAAGTT AGGAGC	290
AR	GAGGAACAGCAGCCT TCACAGCAGC	GCTGCTGCTGAAGAAG TTGCAT	286
DAX-1	GTCCAGGCCATCAAG AGTTTC	CAGCTTTGCACAGAGC ATCTC	418
β-Actin	ATCCCGTGATGAGCAA GTG	AGCTCGATTGACAGTS GAC	540
SGP-1	TAAGGCTAACGAGGA CGTCTGC	GCCTGGACCAGATTCT GCTCAT	484
SGP-2	CATCTGGCATCATAGA CACGCT	ACACAGTGCGGTCATC TTCACC	460
GATA-1	TGTGTGAACTGTGGAG CAACGGC	AAATAGAGGCCGCAGG CATTGCA	362
Testatin	TCTGACTGAACGATGA GG	ACTGTGGAAGTCATGA CG	488
GAPDH	CTGCACCACCAACTGC TTAGC	ATGCCAGTGAGCTTCC GTTC	288

Table 3.3 Primer sequences and product sizes

3.2.4. Steroid treatment of SK11 cells

Steroid treatment of SK11 cells at 33°C and 39°C was carried out by incubation with 10nM and 100nM oestradiol (Sigma) or testosterone (Sigma) for a period of 48 hours. Total RNA was isolated from the cells (section 2.5) and random primed cDNA pools were prepared for Q-RT-PCR (section 2.12.5).

3.2.5. Q-RT-PCR

The relative levels of expression of ERβ and AR in SK11 cells (control/steroid ligand treated and siRNA/shRNA transfected) were determined using quantitative RT-PCR (Taqman) using random primed cDNA (section 3.2.4) as detailed in section 2.12. Results were analysed as in section 2.12.7.

3.2.6. Transient transfections

In order to assess the impact of oestrogenic ligands on the SK11 cells, transient transfections were performed using a reporter construct containing a 3X vitellogenin ERE-linked to TK-luciferase or the androgen-responsive promoter-reporter construct pem-Luc. ERE-Luc was a gift from S.C. Nagel and D.P. McDonnell (Hall and McDonnell, 1999), this vector contains three tandem copies of the vitellogenin ERE sequence (Figure 3.1) ligated into the TK-Luciferase vector (Ligand Pharmaceuticals). Pem-Luc was a gift from Professor G. Verhoeven (Leuven, Belgium). The pem promoter is selectively stimulated by androgens and contains two functional AREs. Both ARE's differ in sequence from the classical steroid response element but exhibited characteristics of direct repeats of the TGTTCT half-site and are preferentially stimulated by androgens (Barbulescu et al., 2001). The sequence of the ARE 1 and 2 from the pem-Luc construct are shown in Figure 3.2.

TAGGTCAcagTGACCTGCGGATCCGCAGGTC
ActgTGACCTAGATCCGCAGGTCActgTGACCT

Figure 3.1 Sequence of the 3x ERE. The 3 repeats of the ERE sequence are underlined

ARE-1	**AGATCTcattcTGTTCC**
ARE-2	**AGCACAtcgTGCTCA**

Figure 3.2 Sequence of the ARE-1 and ARE-2 from the pem-Luc reporter construct. The consensus sequence of each is underlined and the SRE half site variations are shown in blue.

A control vector (pCMV, Promega) was co-transfected in all cases and used to measure Renilla luciferase as an internal control (section 2.4). Transient transfections were carried out and results analysed as in section 2.4.3. All transfections were carried out using serum free media made with DMEM that did not contain phenol red (section 2.13.2)

3.2.6.1. Steroid treatment of SK11 cells following transient transfection

The response of the cells treated with various steroid ligands was investigated. The ligands used were 17βoestradiol, testosterone, the potent oestrogen diethylstilbestrol (DES), the ERβ agonist 3βAdiol (a metabolite of DHT) and the phytoestrogen genistein. The response of two receptor selective ligands was also investigated; PPT™ which is reported to be ERα specific and DPN™ which is reported to be ERβ specific (both of these ligands were supplied by Tocris).

Four hours following transfection with the reporter constructs, complete medium (section 2.13.2) containing either 17βoestradiol, testosterone, 3βAdiol, DES, genistein, DPN™ or PPT™ was added to the cells. Dose response studies were performed by adding the ligands at 5 different concentrations, 10^{-11}M, 10^{-9}M, 10^{-8}M, 10^{-7}M and 10^{-6}M to evaluate the concentration required for maximal ER activation. A control sample was set up in each case, which contained no ligand, only ethanol or DMSO as appropriate. The ligand containing media was replaced again 24 hours following transfection and the cells were harvested 48 hours following transfection for analysis using a luciferase assay (section 2.4) and the results were analysed as in section 2.4.3.

3.2.7. Cloning of ERβ- fluorescent protein construct

In order to visualise the expression of ERβ in the SK11 cells after transient transfection, the full-length murine ERβ cDNA (a gift from Professor M Parker) was amplified and inserted into the dsRed-C2 vector (Clontech).

3.2.7.1. PCR amplification of murine ERβ cDNA

Primers were designed to amplify the coding region of the mouse ERβ cDNA and add a Bgl II restriction enzyme site on the 5' end and a Sac II site on the 3' end (Table 3.4). PCR was carried out using Hot Star Taq polymerase (Qiagen), conditions were as follows:

10x Hot Star Buffer	5μl
20mM dNTPs	1μl
ERβ Sense primer	2μl
ERβ Antisense primer	2μl
Hot Star Taq	0.4μl
H_20	37.6μl

40 cycles of amplification were carried out with an initial annealing temperature of 50°C for 5 cycles followed by 35 cycles at 55°C. The PCR product was run on a 0.8% agarose gel (section 2.7) and the 1.5kb fragment excised using a clean scalpel blade. Gel extraction and PCR product purification was carried out using the Qiagen gel extraction kit according to the manufacturer's instructions as in section 2.8.3.

Sense ERβ cloning primer 5'→3'	Antisense ERβ cloning primer 5'→3'
GCAATTAGATCT**ATG**ACATTCTACA GTCCTGCTGT	GCGCCCGCGG**TCA**CTGTGACTG GAGGTTCTG

Table 3.4 Sequence of primers used to amplify murine ERβ cDNA with restriction enzyme sites at the 5' and 3' ends to allow directional cloning into the dsRed vector. Bgl II site is shown in red, Sac II site is shown in blue. The start (ATG) and stop (TCA) sites of the ERβ cDNA are underlined in bold.

3.2.7.2. Topo-TA cloning of ERβ PCR product

The gel extracted and purified ERβ PCR product was cloned into the TOPO-pCRII vector (Invitrogen) according to manufacturer's instructions to facilitate sequencing of the PCR product. Briefly, 100ng of PCR product was mixed with 1μl salt solution (Invitrogen), 1μl TOPO vector and 2μl sterile H_20. The mixture was incubated at room temperature for 10 minutes before 2μl of the mix was transformed (section 2.8.5) into 50μl XL-1 Blue cells (Stratagene). The entire transformation reaction was spread onto LB Agar plates containing ampicillin at a final concentration of 50μg/ml and incubated inverted overnight at 37°C. Individual colonies were picked the following day and used to inoculate 10mls of LB-broth containing ampicillin which were incubated overnight in a rotary shaker at 37°C, 225 rpm. The next day, mini-preps were performed using the Promega Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions (section 2.8.6). Restriction enzyme digestion (section 2.8.2) followed by agarose gel electrophoresis (section 2.7) was carried out to check that the plasmid contained the insert of the correct size. Sequencing (section 2.9) was performed to ensure no errors had been introduced into the sequence before glycerol stocks (section 2.9.1) and large-scale CsCl preparations were made (section 2.10).

3.2.7.3. Ligation into dsRed vector

The ERβ cloned into the TA cloning vector was digested with Bgl II and SacII in order to release the ERβ fragment from the vector. The dsRed vector was digested

with the same enzymes to allow ligation. Digests were set up as in section 2.8.2 and the vector dephosphorylated to ensure no re-ligation occurred using SAP (Promega, section 2.8.2). Ligation of the ERβ insert into the dsRed vector was carried out as in section 2.8.4 using T4 DNA ligase (NEB), incubated overnight at 16°C. Transformation of XL-1 blue competent cells with the ligation product was carried out as in section 2.8.5 and the resulting colonies were picked, grown up and miniprepped as in section 2.8.6. Sequence analysis was performed to check the vector contained the correct insert before glycerol stocks and a large scale CsCl preparation was prepared (sections 2.9.1 and 2.10).

3.2.8. RNA interference

3.2.8.1. Design and construction of siRNA's

Linear siRNA's were designed as described in section 2.11.1. Table 3.5 shows the sequences of the siRNA's used in this chapter. A control siRNA to GAPDH provided by Ambion and a siRNA to GFP were both synthesised using the Silencer™ siRNA construction kit (Ambion) as described in section 2.11.2. Control siRNA sequences for α-tubulin, β-tubulin and lamin A/C, which have been reported to produce high levels of knockdown in mammalian cells, were selected from a database of sequences published on the Dharmacon website (www.dharmacon.com). Sequences for GFP and murine ERβ were chosen using the design guidelines provided on the Ambion website (www.ambion.com) and the ERβ siRNA was synthesised by MWG.

Targeted Gene	siRNA target sequence
ERβ	AAGAAGAUAAUGGUCAAGCUU
β-tubulin	AAGACAGAGCCAAGUGGACUC
α-tubulin	AAGAUAUUGAGCGUCCAACCU
Lamin A/C	AAGAAGCAGCUUGACGAUGAG
GFP	AAGCTGACCCTGAAGTTCATC
GAPDH	No sequence supplied

Table 3.5 Sequence of siRNA's

3.2.8.2. Fluorescent labelling of siRNAs

Fluorescently labelled siRNAs were synthesised and used to check whether they were being successfully transfected into the cells. By labelling the siRNA duplex with fluorescein, the route of the siRNA into the cell can be tracked by using confocal microscopy. The Silencer ™ siRNA labelling kit from Ambion was used according to the manufacturer's instructions. The labelling reaction was prepared as follows:

Nuclease free water	18.3µl
10x labelling buffer	5.0µl
21mer siRNA duplex at 20µM	19.2µl
FAM labelling reaction	7.5µl

The samples were incubated in the dark at 37°C for 1 hour then precipitated using ethanol; 5µl 5M NaCl and 125µl 100% cold ethanol was added to the labelling reaction and mixed well. The mixture was placed at -20°C for 1 hour to precipitate the labelled RNA. The RNA was centrifuged at 13,000 rpm for 20 minutes to pellet the RNA, and a green coloured pellet was formed. The supernatant was removed and 175µl cold 70% ethanol was added to the pellet and the sample centrifuged for 5 minutes at 13,000 rpm. The ethanol was removed and the pellet air-dried for 5-10 minutes before being resuspended in 19.2 µl of nuclease free water. This resulted in the same nucleic acid concentration as at the beginning of the reaction. The RNA was quantified using the GeneQuant spectrophotometer (section 2.10.1) before being used for transfection.

3.2.8.3. Transfection of siRNA's using a fluorescently labelled reagent

JetSI–FluoR (Polytransfection) is a rhodamine-conjugated transfection reagent that facilitates the measurement of transfection efficiency and the tracking of siRNA complexes. Optimisation of the transfections showed that the best transfection efficiency was seen using 100nM siRNA.

The following calculation was used to determine the appropriate volume of jetSI-FluoR:

Amount of siRNA (ng) x 0.003 = Xμl jetSI FluoR

For transfection of a 3.5cm glass bottomed dish using 100nM siRNA, 20μl of siRNA was added to 80μl of serum-free medium in a sterile 1.5ml tube. In a separate tube, 8.4μl jetSI-FluoR was added to 91.6 μl serum-free medium. The tubes were vortexed vigorously and incubated at room temperature for 10 minutes. The jetSI FluoR mix was then added to the siRNA mix, vortexed for 10 seconds and incubated for 30 minutes in the dark. The JetSI/siRNA mixture was added to the wells, which already contained 1ml of serum free transfection medium and the cells incubated for 4 hours. After 4 hours, 1ml of complete medium was added to the cells and they were returned to the incubator. Cells were observed on the confocal microscope after 24 and 48 hours.

3.2.8.4. Transfection of siRNA's into SK11 cells

Routine transfection of siRNA's was performed using oligofectamine as described in section 2.11.3.1. Ligand addition, if required, was performed 4 hours after transfection and gene activity was measured 24-48 hours after transfection by luciferase assay (section 2.4), confocal analysis (section 2.11.4), Q-RT-PCR (section 2.12), or immunohistochemistry (section 3.2.8.5)

3.2.8.5. Immunohistochemistry of siRNA transfected cells

Cells were grown on 2 well glass chamber-well slides or 3.5cm glass bottomed dishes and transfected with siRNA's (section 3.2.8.4). Cells were fixed using ice-cold methanol or modified Davidson's fixative (section 2.13.1) before immunohistochemistry was performed as described in section 2.2.10. Cells were visualised using a LSM 510 confocal microscope as described in section 2.11.4. Table 3.6 gives details of the antibodies used.

Primary antibody	**Dilution**	**Species raised in**	**Supplier**
Tyrosine α- tubulin	1:2000	Mouse	Sigma
β-tubulin	1:1500	Mouse	Sigma
Lamin A/C	1:500	Mouse	Santa-Cruz
Secondary antibody	**Dilution**	**Species raised in**	**Supplier**
Goat anti-mouse Alexa fluor 546	1:200	Goat	Molecular probes
Goat anti-mouse Cy-5	1:60	Goat	Molecular probes

Table 3.6 Antibodies used in fluorescent immunohistochemistry

3.2.9. Preparation of plasmid vectors containing shRNA's

Section 2.11.5 describes in detail the design and construction of shRNA containing plasmid vectors. The siRNA specific for ERβ is shown in Table 3.5. Table 3.7 shows the sequence of the DNA sense and antisense oligonucleotides used in the construction of the shERβ which was inserted into pSilencer.

Sense oligonucleotide 5'→ 3'	**Antisense oligonucleotide 5'→ 3'**
GATCCAAGAAGATAATGGTCA	AGCTTTTCCAAAAAAGAAGATAA
AGCTTCTCAAGAGAAAGCTTG	TGGTCAAGCTTTCTCTTGAGAAG
CCATTATCTTCTTTTTTGGAAA	CTTGACCATTATCTTCG

Table 3.7 ERβ shRNA oligonucleotide sequence. The 19mer ERβ sequence specific siRNA sequence (sense and antisense) is underlined and shown in red with the loop sequence shown in blue.

Transfection of the shERβ pSilencer vector was performed as in section 3.2.8.4 and subsequent analysis of knockdown by confocal microscopy, luciferase assay and Taqman quantitative RT-PCR was performed as described in sections 2.11.4, 2.4 and 2.12 respectively.

3.3. Results

3.3.1. Immunolocalisation of ERα, ERβ and AR in the immature and adult mouse testis

ERα was immunolocalised to the Leydig cells of the immature and adult mouse testis. No immunoexpression was detected in the Sertoli cells or the germ cells (Figure 3.3, panels A and B).

ERβ protein was localised to the nuclei of the germ cells of both the immature and adult testes. The Sertoli cells and the Leydig cells also stained positive for ERβ (Figure 3.3, panels C and D).

AR protein was localised to the nuclei of the Sertoli cells, the Leydig cells and the peritubular myoid cells of the immature and adult testis (Figure 3.3, panels E and F). The Sertoli cell expression is weaker in the 10-day-old section than in the adult section; in the adult, expression was stage dependent. No AR positive germ cells were observed in the immature or the adult testis.

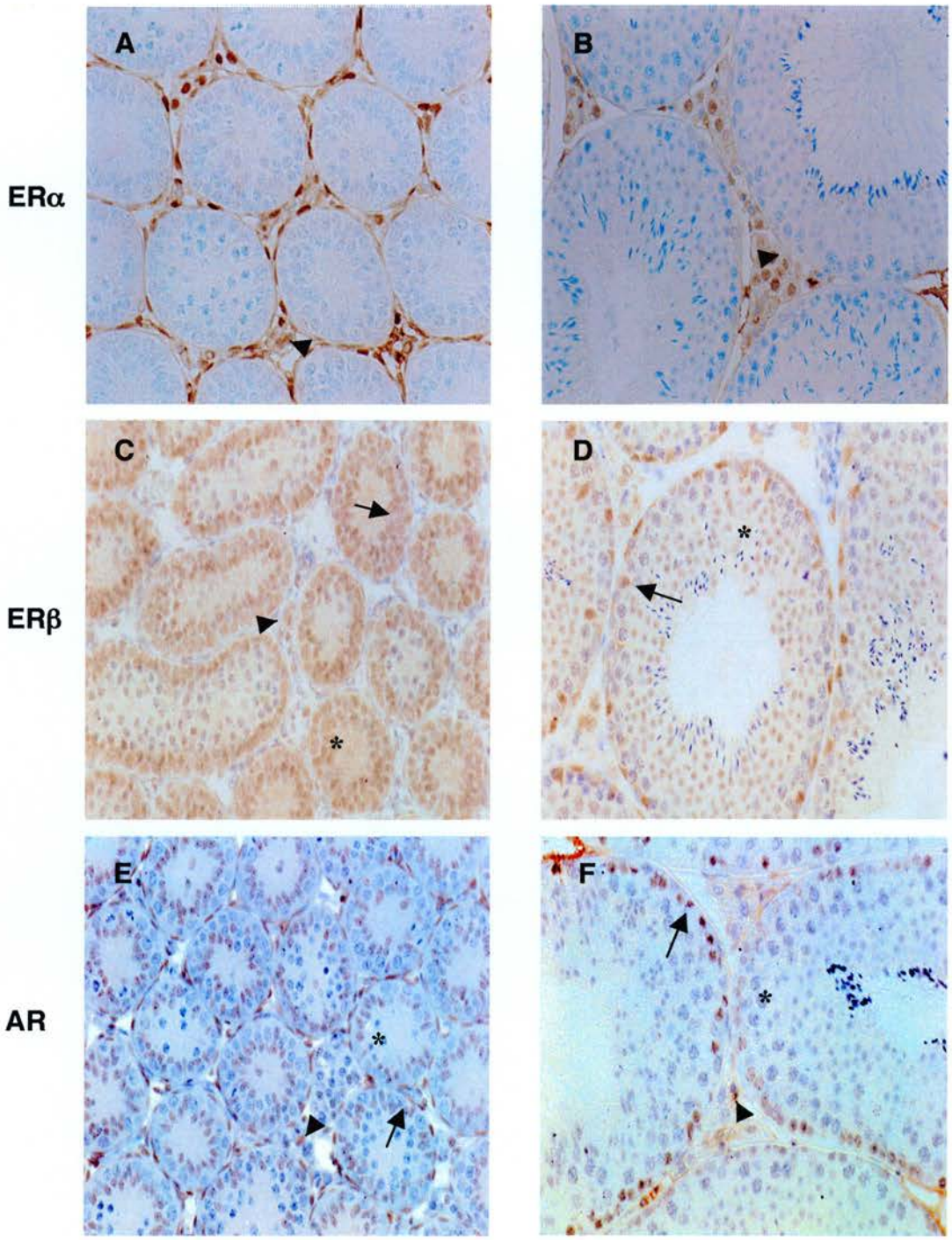


Figure 3.3 Immunohistochemical analysis of ERα, ERβ and AR protein expression in the mouse testis. Sections taken from a 10-day-old mouse were stained for ERα (A), ERβ (C) and AR (E). Adult testis sections were stained for ERα (B), ERβ (D) and AR (F). Arrows represent Sertoli cells, arrowheads represent Leydig cells and asterisks represent germ cells.

3.3.2. Characterisation of the SK11 cell line

The SK11 cell line was first characterised in 1995 and therefore before embarking on a large series of experiments, the pattern of gene expression was checked to confirm that it had maintained its phenotype. It has been reported that freshly isolated Sertoli cells lose their expression steroid receptors and Sertoli cell products (Karl and Griswold, 1990)

3.3.2.1. Differentiation of the SK11 cell line

Differentiated (39°C) and undifferentiated (33°C) cells stained with β-actin are shown in Figure 3.4. Undifferentiated cells are shown in panels A and B. The cells are large with an even distribution of β-Actin. After differentiation, the cells became more rounded and flattened in shape and showed an accumulation of β-actin around their nuclei (Figure 3.4, panels C and D). The apparent increase in β-actin observed in the differentiated cells was mirrored by an increase in mRNA levels as detected by semi-quantitative RT-PCR (Figure 3.5, lanes 3 and 4). RT-PCR was performed on the same samples using primers for GAPDH, a housekeeping gene, the levels of which should remain constant throughout, acting as a control.

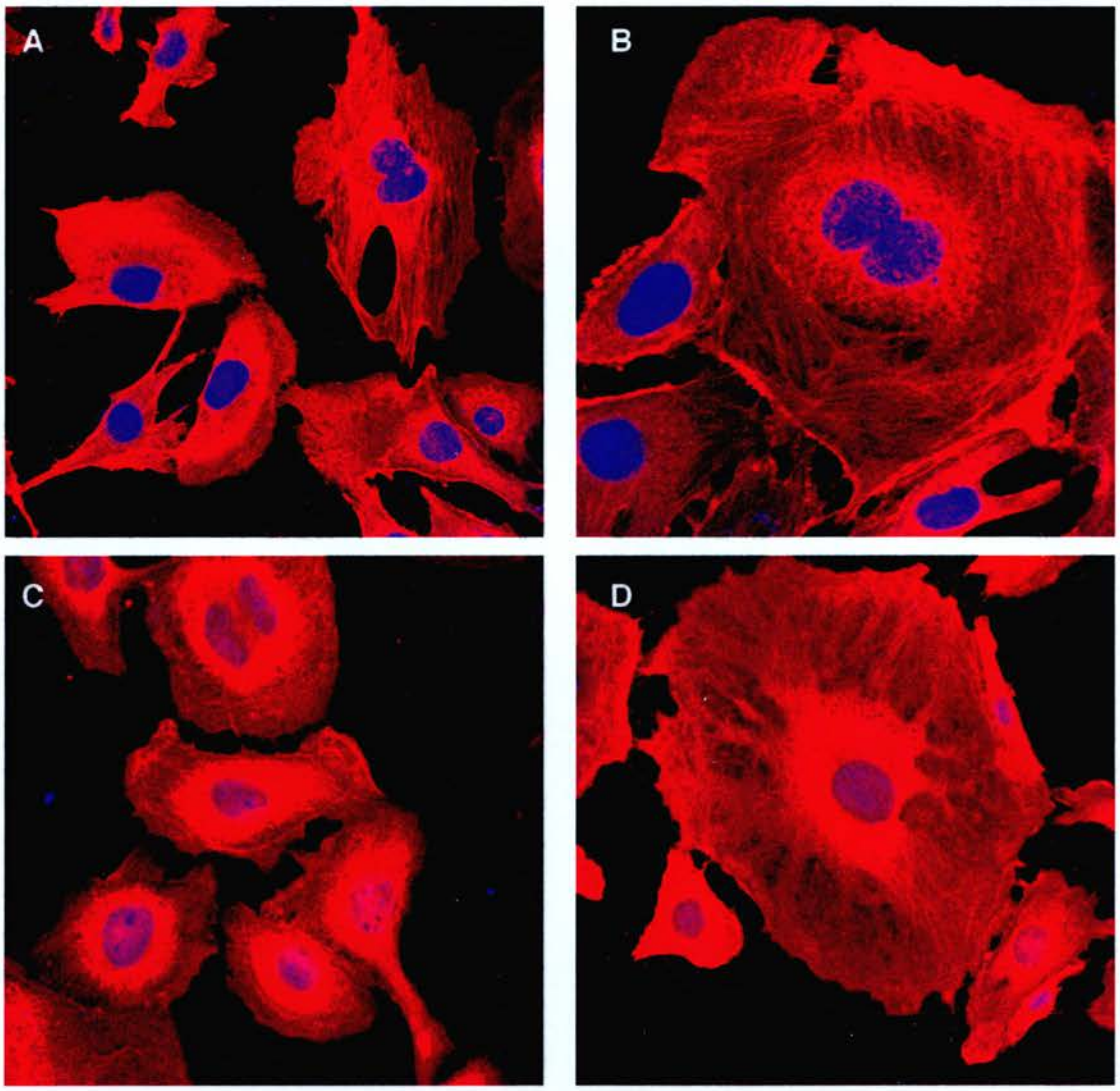


Figure 3.4 Confocal image of the SK11 Sertoli cell line growing at 33°C (A and B) and after culture at 39°C for 48 hours (C and D) after immunostaining for β-Actin (red) to show changes in morphology after differentiation. Cells are counterstained using To-Pro3 (blue).

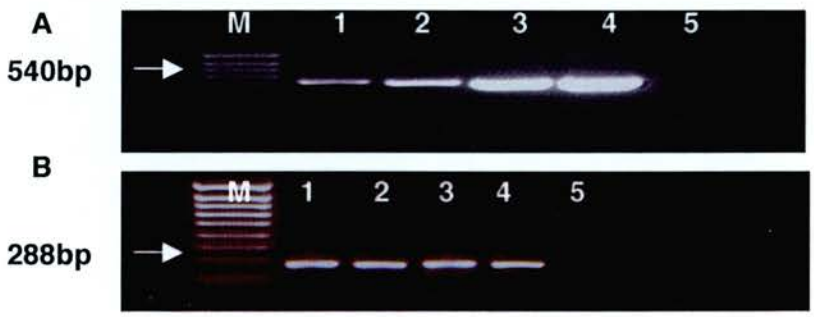


Figure 3.5 Panel A shows β-actin mRNA expression in d10 testis (lane 1), adult testis (lane 2), undifferentiated SK11 cells (lane 3) and differentiated SK11 cells (lane 4). Panel B shows GAPDH expression in the same samples M represents DNA hyperladder.

3.3.2.2. Expression of Sertoli cell product mRNA's in SK11 cells

Expression of mRNA's previously identified as Sertoli cell specific products was investigated in SK11 cells by RT-PCR using specific primers designed for GATA-1, SGP-1, SGP-2, testatin and DAX-1 (Figure 3.6 and 3.7). cDNA's prepared from d8 and adult testes were used as controls.

As expected, mRNA's for SGP-1 and SGP-2 (Figure 3.6) were both detected in the adult mouse testis. Both mRNA's were expressed in the SK11 cells; SGP-1 was expressed in higher amounts in the differentiated sample. No change was observed in SGP-2 mRNA expression in the differentiated compared to the undifferentiated cells. Messenger RNA for GATA-1 appeared lower in the SK11 cells than in the intact testis but no difference was observed between undifferentiated and differentiated cells. Testatin and Dax-1 mRNA's were expressed in similar amounts in both undifferentiated and differentiated cell types (Figure 3.7).

3.3.2.3. Expression of Sertoli cell specific proteins in undifferentiated and differentiated SK11 cells

Immunohistochemistry of undifferentiated and differentiated SK11 cells was carried out to confirm that the SK11 cells had actively translated the mRNA's detected by RT-PCR (Figure 3.8). Higher levels of SGP-1 expression was seen in the differentiated SK11 cells compared to the undifferentiated cells. GATA–1 protein expression was similar in both cell types.

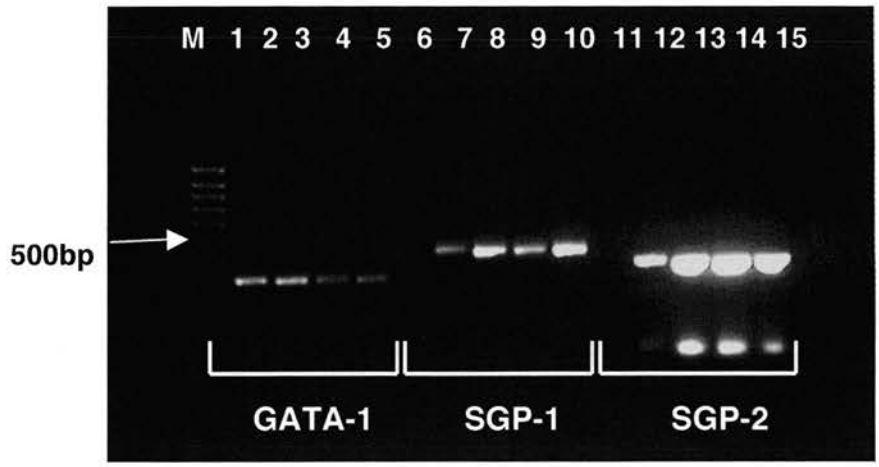


Figure 3.6 Expression of GATA-1 (362bp) SGP-1 (484 bp) and SGP-2 (460bp) in testis and SK11 cells. Lanes 1, 6 and 11 are adult whole testis cDNA samples. Lanes 2, 7 and 12 are d10 mouse testis cDNA samples. Lanes 3, 8 and 13 are undifferentiated SK11 cells cultured at 33°C. Lanes 4,9 and 14 are differentiated SK11 cells cultured at 39°C. Lanes 5,10 and 13 are –ve control samples and M represents Hyperladder 1 DNA marker.

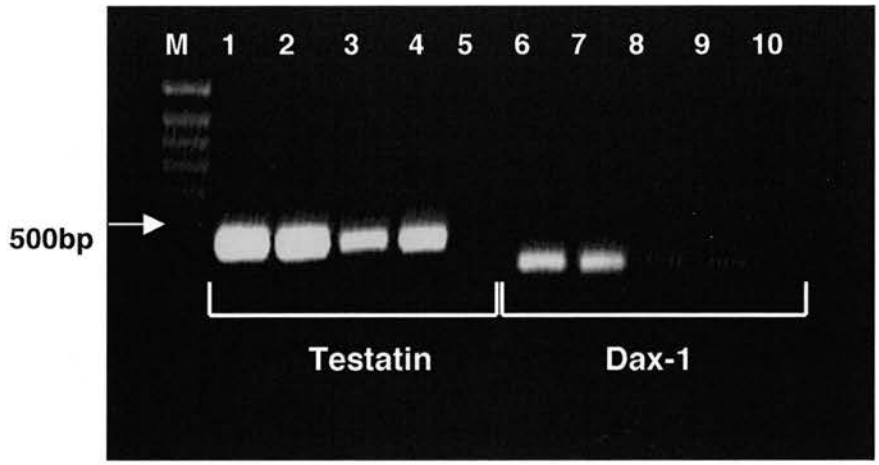


Figure 3.7 Expression of Testatin (488bp) and Dax-1 (418bp) in mouse testis and SK11 cells. Lanes 1 and 6 are adult whole testis cDNA samples. Lanes 2 and 7 are d10 mouse testis cDNA samples. Lanes 3 and 8 are undifferentiated SK11 cells cultured at 33°C. Lanes 4 and 9 are differentiated SK11 cells cultured at 39°C. Lanes 5 and 10 are –ve control samples and M represents Hyperladder 1 DNA marker.

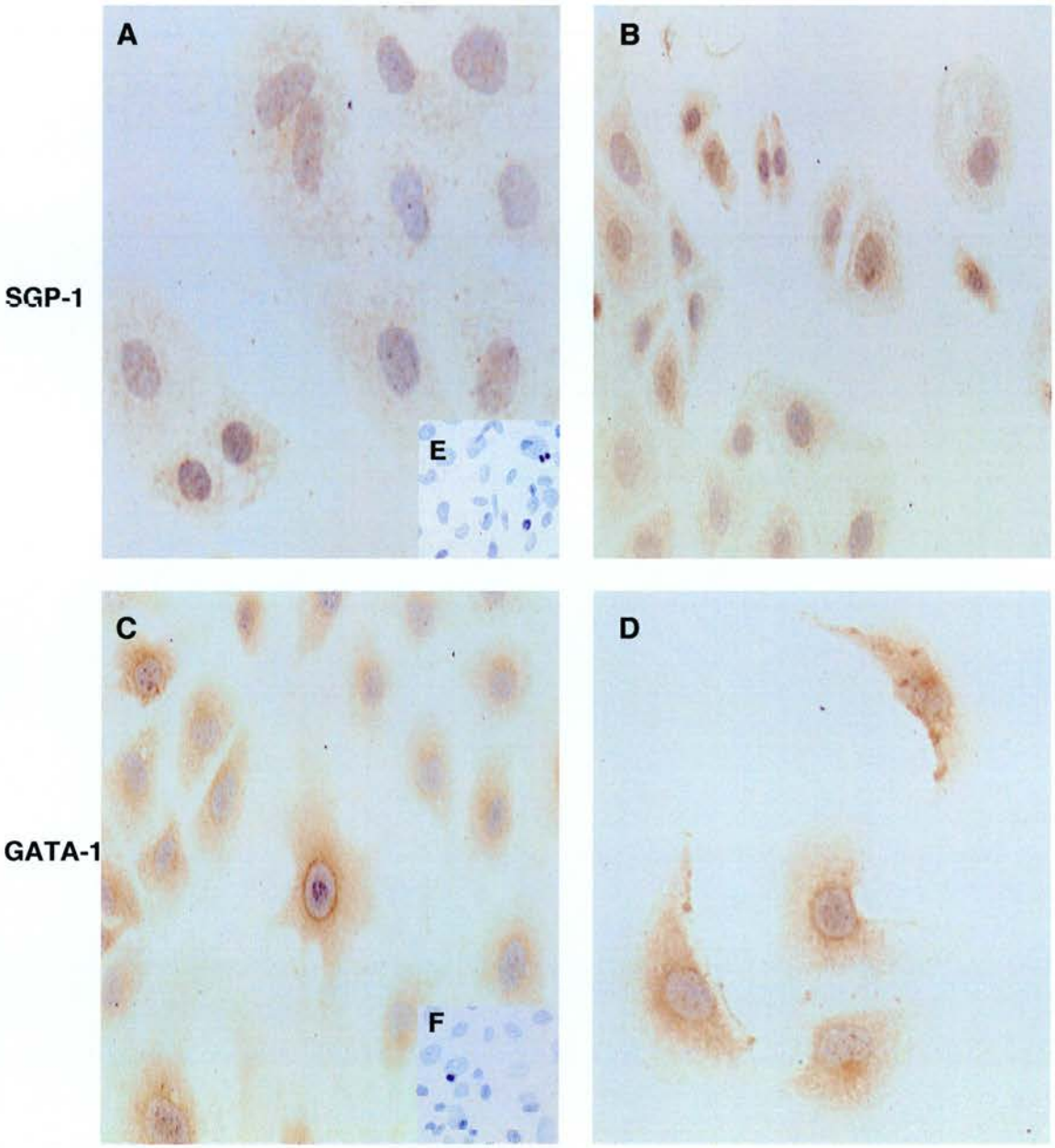


Figure 3.8 Immunohistochemical analysis of SGP-1 and GATA-1 protein expression in undifferentiated and differentiated SK11 cells. SK11 cells cultured at 33°C were stained for SGP-1 (A) and GATA-1 (C). SK11 cells cultured at 39°C stained for SGP-1 (B) and GATA-1 (D). E and F show SK11 cells stained with the respective secondary antibody only, as a negative control.

3.3.3. Expression of steroid hormone receptors in SK11 cells

As the results from the previous sections showed that the SK11 cells were behaving in a similar way to Sertoli cells in vivo, with respect to their expression pattern, the following experiments were performed in order to investigate the expression of steroid hormone receptor mRNA's and protein within the cells.

3.3.3.1. Steroid hormone receptor mRNA expression

Following PCR, DNA products corresponding to ERα (290bp), ERβ (339bp) and AR (266bp) were amplified from cDNA's prepared from adult mouse testis (Figure 3.9). cDNA's prepared from SK11 cells (both differentiated and undifferentiated) were positive for ERβ (Figure 3.9) but no ERα expression was detected (Figure 3.9). Levels of expression of ERβ mRNA's were similar in differentiated and undifferentiated cells. AR specific mRNA's were detected in SK11 cells; levels of AR mRNA appear higher in differentiated cells compared to the undifferentiated cells (Figure 3.9).

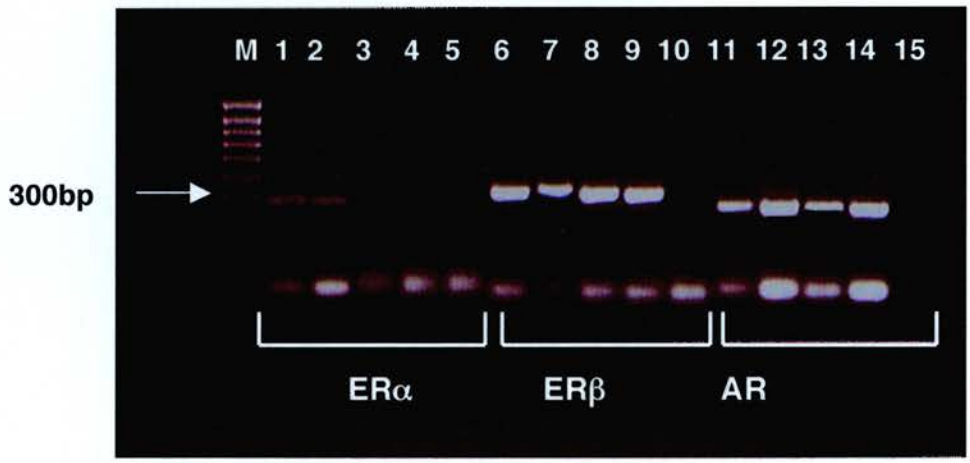


Figure 3.9 RT-PCR using primers specific for ERα (290 bp), ERβ (339bp) and AR (286bp). Lanes 1.6 and 11 are 8d testis samples, lanes 2,7 and 12 are adult testis samples, lanes 3,8 and 13 are undifferentiated SK11 cells, lanes 4,9 and 14 are differentiated SK11 cells and lanes 5,10 and 15 are H_20 –ve control samples. M represents Hyperladder 1 DNA marker.

3.3.3.2. Steroid hormone receptor protein expression

Immunohistochemistry performed on the cell monolayers resulted in the detection of ERβ and AR protein but not ERα in the SK11 cells (Figure 3.10). ERβ (Figure 3.10, panels C and D) and AR (Figure 3.10, panels E and F) was observed within the nuclei of the cells. More intense AR staining was immunolocalised in the differentiated cells cultured at 39°C compared to the undifferentiated cells. No difference was observed in the intensity of ERβ immunostaining in differentiated and undifferentiated SK11 cells. Expression of ERα protein was not observed in the cells either in the differentiated or undifferentiated states (Figure 3.10, panels A and B).

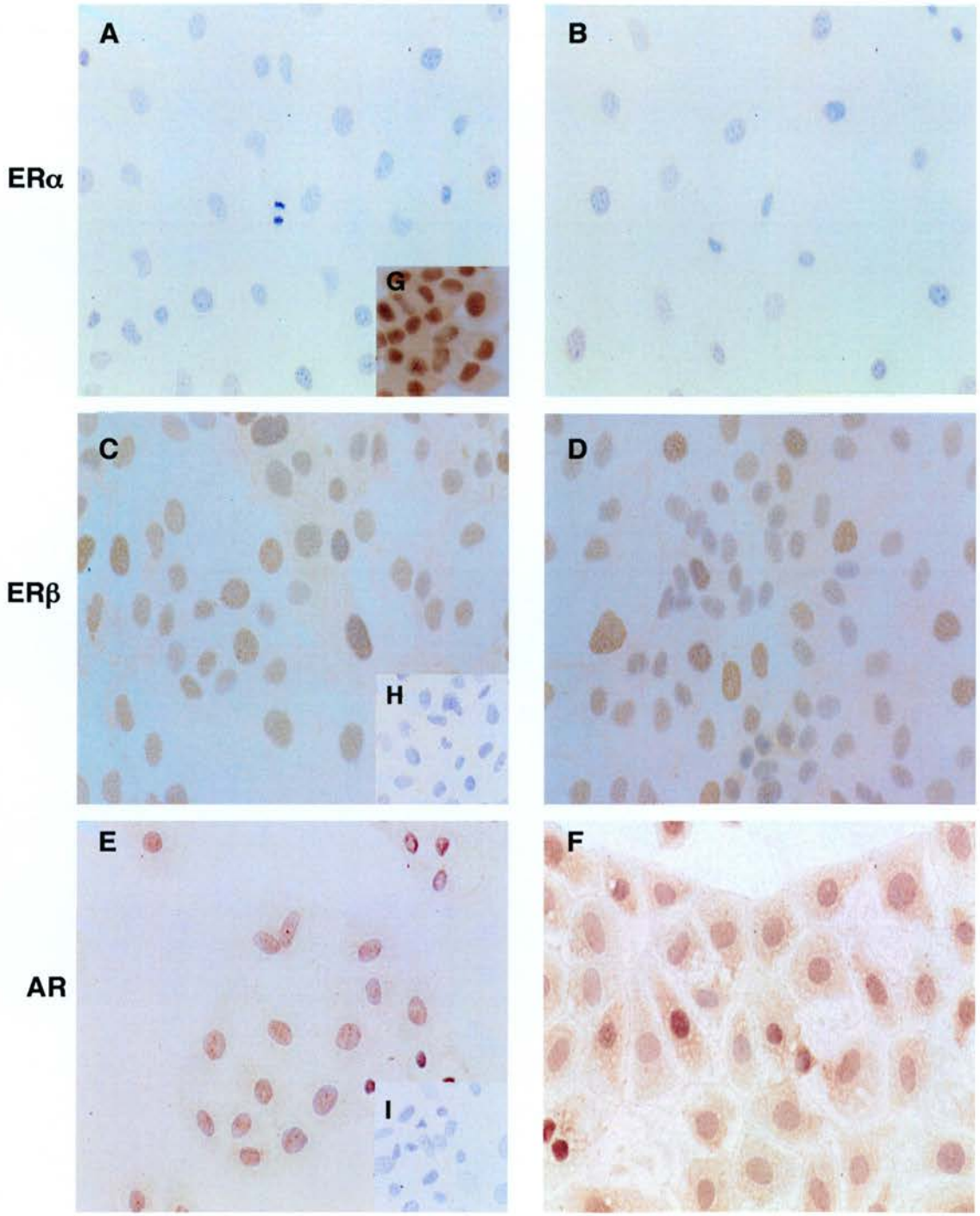


Figure 3.10 Immunohistochemical analysis of ERα, ERβ and AR protein expression in undifferentiated and differentiated SK11 cells. SK11 cells cultured at 33°C were stained for ERα (A), ERβ (C) and AR (E). SK11 cells cultured at 39°C stained for ERα (B) and ERβ (D) and AR (F). Small inset image in A shows MCF-7 cells immunostained using the same antibody against ERα, as a positive control. G, H and I show SK11 cells immunostained using only the secondary antibodies, acting as a negative control.

3.3.4. Steroid responsiveness of SK11 cells

As the cells were shown to express both ERβ and AR (section 3.3.3.1 and 3.3.3.2), the following experiments were designed and carried out to investigate whether the cells were able to respond to treatment with oestrogens and androgens.

3.3.4.1. Impact of steroid hormones on expression of steroid hormone receptor mRNA's

Expression of ERβ and AR mRNA's in undifferentiated SK11 cells were analysed by TaqMan after treatment with either 10nM and 100nM 17β oestradiol or testosterone (Figure 3.11 A and B). Compared to a control sample, which was treated with vehicle alone (ethanol), addition of oestradiol resulted in a significant increase in ERβ mRNA expression ($P < 0.001$). A 19-fold increase of ERβ mRNA expression occurred after exposure to 10nM oestradiol. When cells were exposed to testosterone, no increase in expression of ERβ was observed at either concentration used.

Addition of oestradiol had no effect on AR expression in the cells but treatment with testosterone at both concentrations resulted in a 4-fold and 14-fold increase in AR mRNA levels at 10nM and 100nM testosterone respectively.

The same experiment was repeated this time using cells that have been differentiated by culture at 39°C (Figure 3.11 C and D). Significantly increased ERβ mRNA expression was observed upon addition of oestradiol however, the maximum response was seen at 100nM E and the fold increase was not as great (13.8 fold) as was seen in the undifferentiated cells. Addition of testosterone had no effect on ERβ levels in the SK11 cells. Androgen receptor expression increased with addition of testosterone at both concentrations with a 14-fold increase at 10nM T and a 33-fold increase at 100nM T being seen. This was higher than that observed in the undifferentiated cells. A slight effect was observed on androgen receptor expression upon addition of oestradiol with a 3-fold increase being observed but this increase was not shown to be statistically significant.

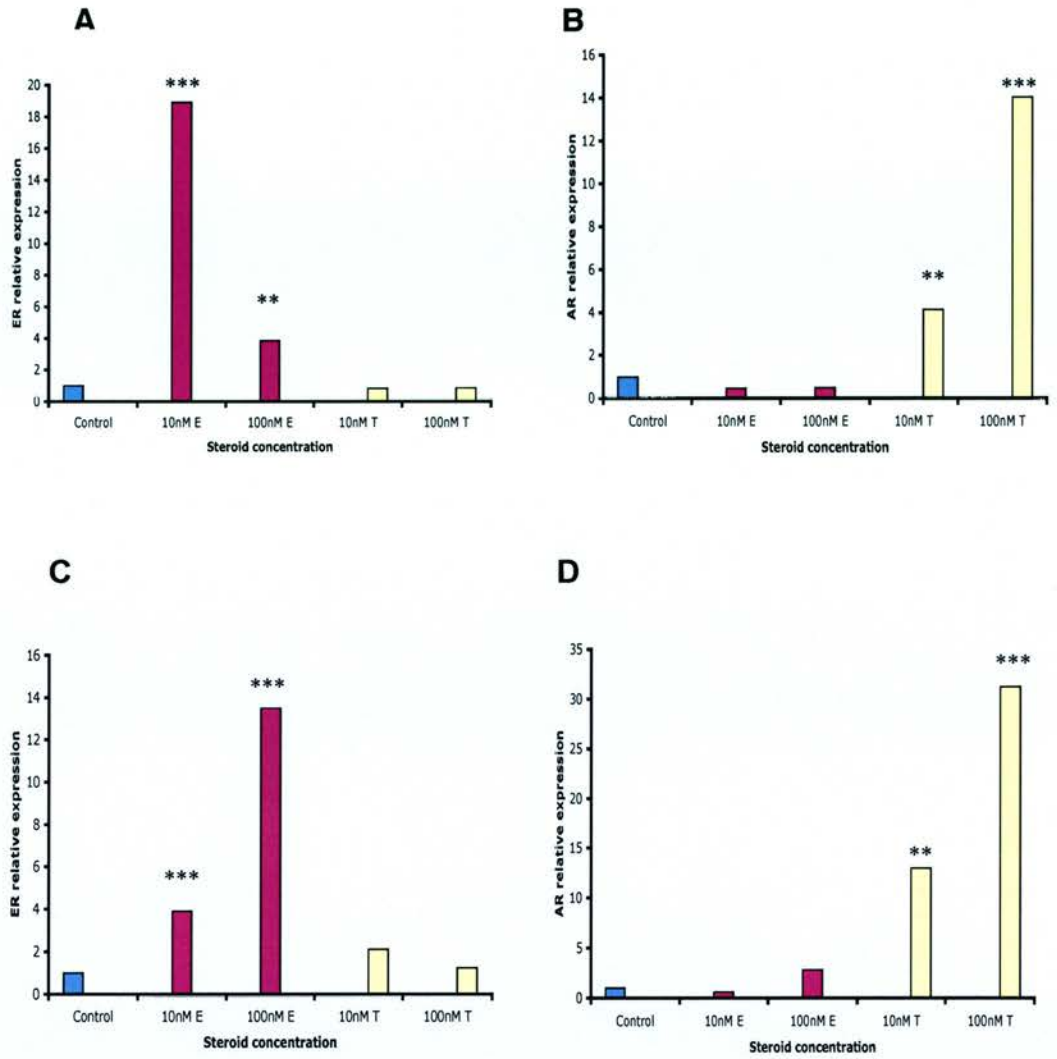


Figure 3.11 Steroid responsiveness in the SK11 cell line, both undifferentiated cells cultured at 33°C (A and B) and differentiated cells cultured at 39°C (C and D). A and C show ERβ relative amounts, C and D show AR relative amounts determined by Taqman Q-RT-PCR after culture with oestradiol (purple bars) and testosterone (yellow bars) at a concentration of either 10nM or 100nM. Results are shown as relative expression compared to a vehicle treated control (blue bars). Statistical significance was calculated using one-way ANOVA, n=2. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3.4.2. Activation of an oestrogen response element reporter in SK11 cells

In order to investigate whether the ERβ protein detected in the SK11 cells was functional, cells were co-transfected with an ERE-luciferase reporter plasmid and the Renilla luciferase internal control plasmid. Oestrogenic ligands (natural and synthetic) were added to the cells 4 hours post transfection to obtain a dose response. The results were normalised and expressed as a fold increase compared to a no ligand control. One-way ANOVA was performed on the data to test for statistical significance.

3.3.4.2.1. Natural oestrogenic ligands

E_2 and 3βAdiol are both natural ligands synthesised within the testis. A dose response curve was observed when E_2 was added in increasing concentrations (10pM to 1μM) to the SK11 cells transiently transfected with the 3x ERE-Luc reporter construct (Figure 3.12A). The maximum response was seen with addition of 100nM E_2. A dose dependant response was also observed after ERE transfection when 3βAdiol was added in increasing concentrations (10pM to 1μM) with the maximum response was seen with addition of 1μM 3βAdiol (Figure 3.12A).

3.3.4.2.2. Phytoestrogens

Genestein is a phytoestrogen, which is reported to activate ERβ. SK11 cells transfected with ERE-Luciferase and stimulated with genistein resulted in a dose dependant increase of ERE activity with maximum response at 1μM genistein (Figure 3.13).

3.3.4.2.3. Synthetic ligands

Three synthetic oestrogenic ligands were used to stimulate the cells after transient transfection. DES which is reported to activate both ERα and ERβ, DPN which is an ERβ selective ligand and PPT which is reported to be ERα selective. When transiently transfected SK11 cells were treated with increasing concentrations of DES (Figure 3.15), the response was slight at low concentrations (10pM-10nM). At higher concentrations, (100nM and 1μM) the ERE is activated to a greater extent

with a 5.8 and 6.5 fold increase being observed at 100nM and 1μM DES respectively.

The ERE was activated in a dose dependant manner when DPN™ was added in increasing concentrations (10pM to 1μM). The maximum response was seen with addition of 100nM DPN™ (Figure 3.14A).

No activation of the ERE was observed after addition of PPT™ in increasing concentrations (10pM to 1μM) after transient transfection (Figure 3.14B).

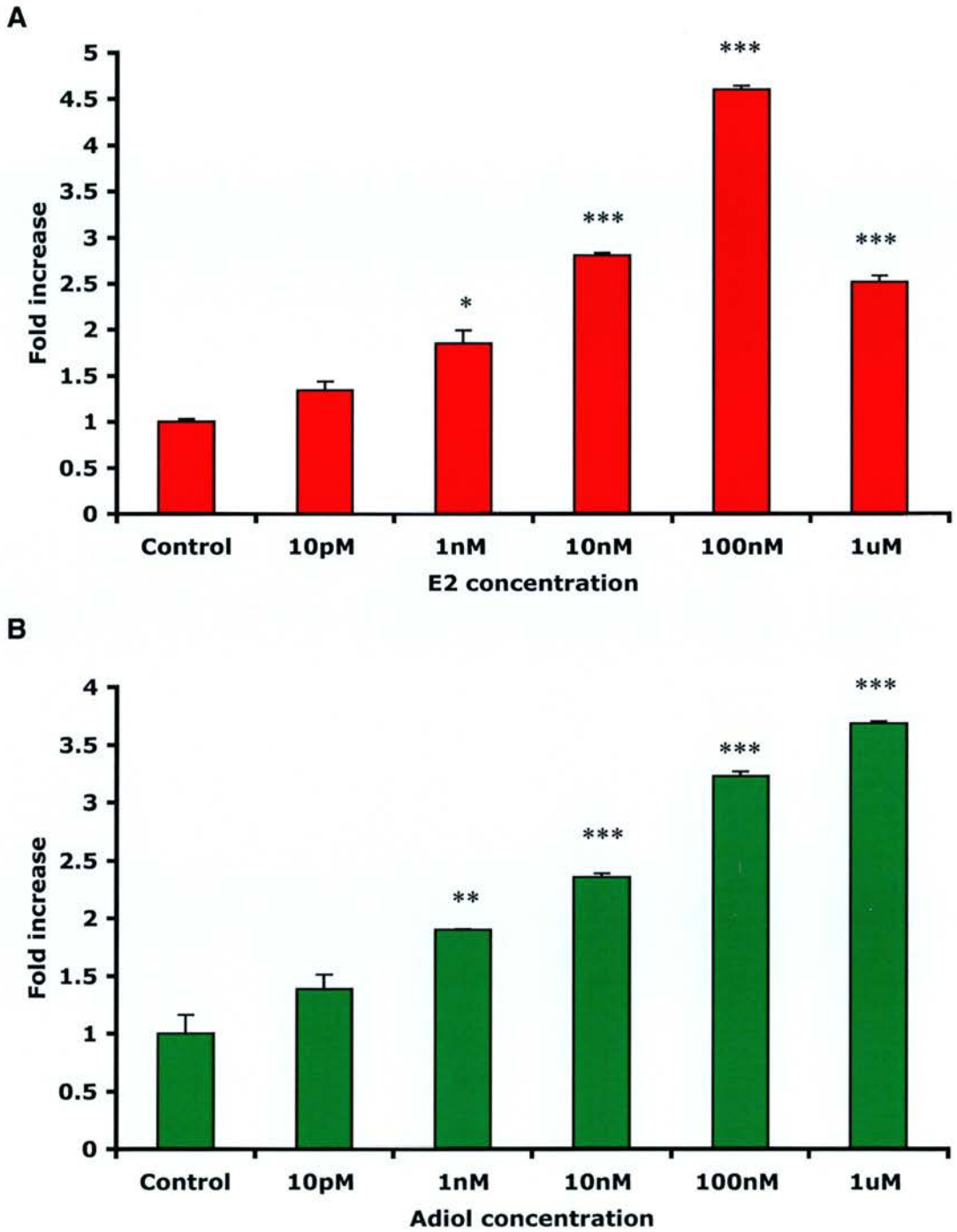


Figure 3.12 Dose response in SK11 cells after ligand treatment. SK11 cells were transiently transfected with the 3x ERE-Luc reporter and the pRL-CMV Renilla internal control. Cells were treated with a vehicle, or increasing concentrations of (A) 17β oestradiol (10pM to 1μM) or (B) 3β Adiol (10pM to 1μM). Results are expressed as ± SEM (n=3). Statistical analysis was performed using one-way ANOVA, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

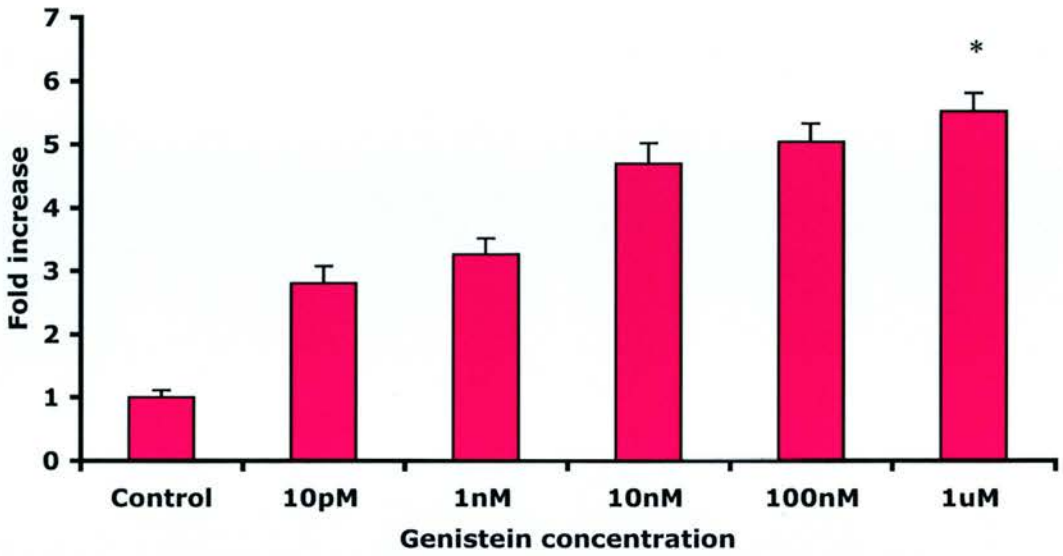


Figure 3.13 Dose response in SK11 cells after genistein treatment. SK11 cells were transiently transfected with the 3x ERE-Luc reporter and the pRL-CMV Renilla internal control. Cells were treated with a vehicle, or increasing concentrations of genistein (10pM to 1μM). Results are expressed as ± SEM (n=3). Statistical analysis was performed using one-way ANOVA, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

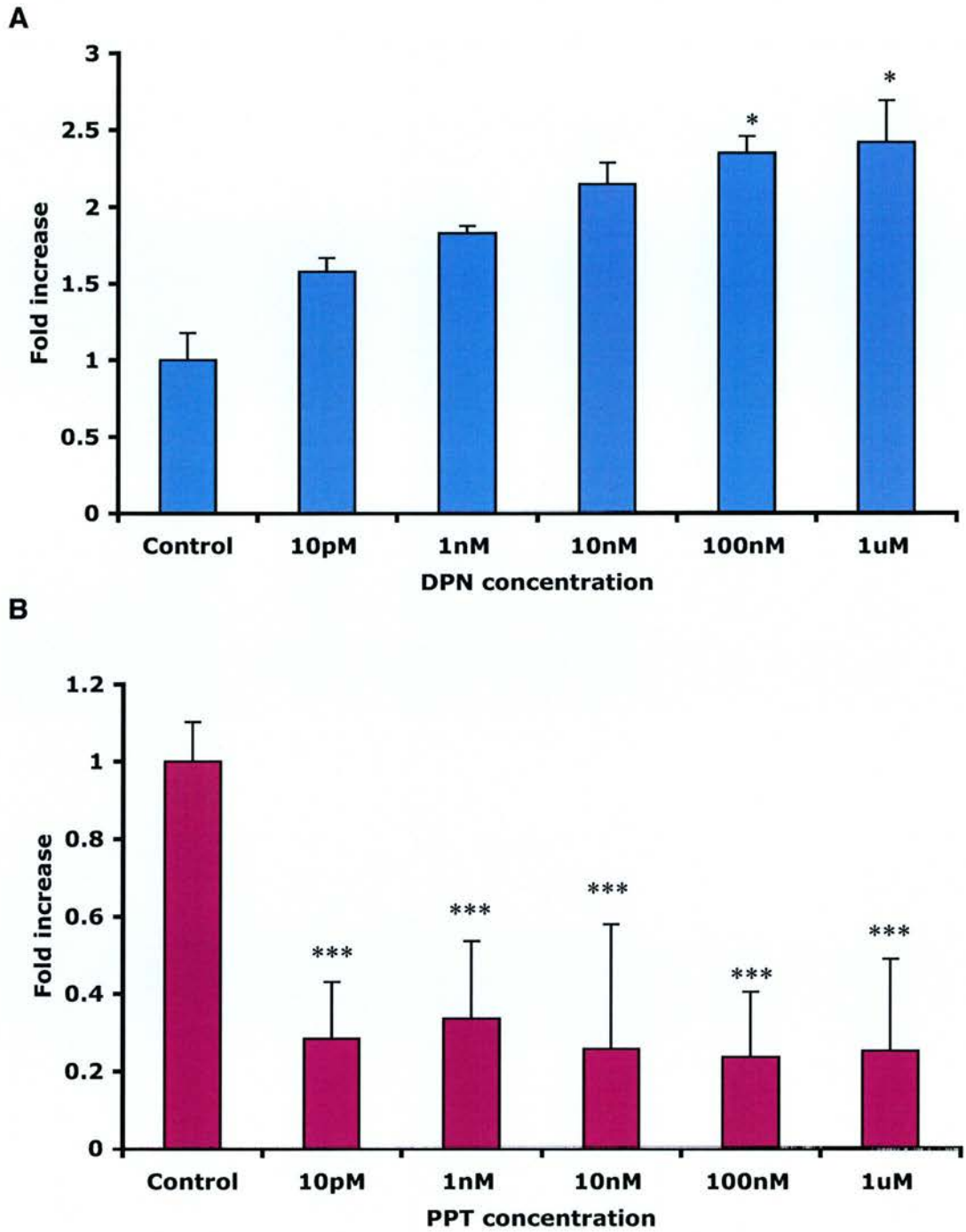


Figure 3.14 Dose response in SK11 cells after ligand treatment. SK11 cells were transiently transfected with the 3x ERE-Luc reporter and the pRL-CMV Renilla internal control. Cells were treated with a vehicle, or increasing concentrations of (A) DPN™ (10pM to 1μM) or (B) PPT™ (10pM to 1μM). Results are expressed as ± SEM (n=3). Statistical analysis was performed using one-way ANOVA, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

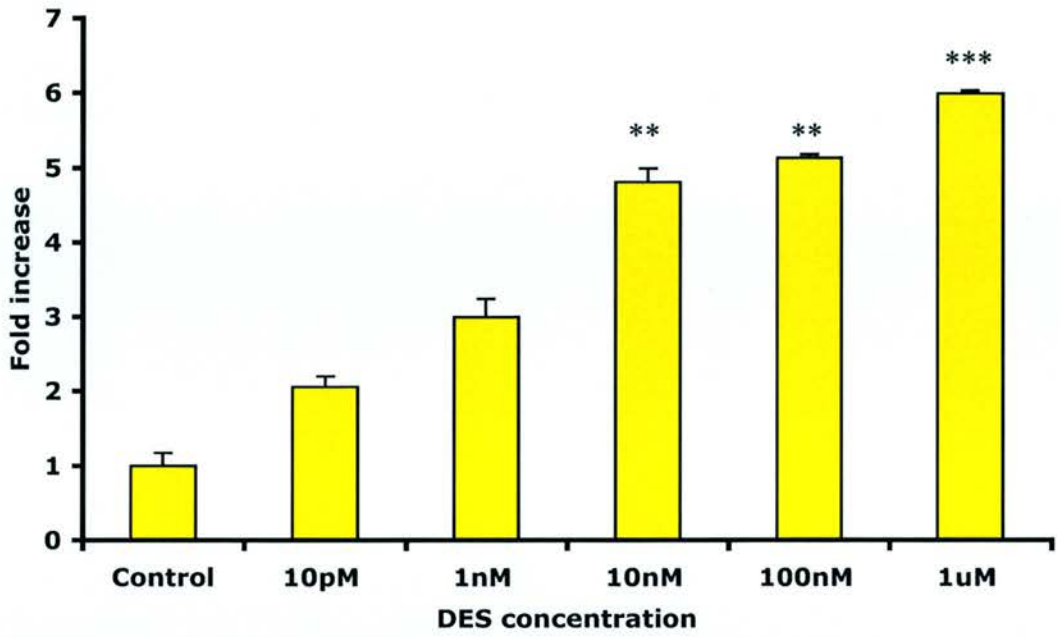


Figure 3.15 Dose response in SK11 cells after DES treatment. SK11 cells were transiently transfected with the 3x ERE-Luc reporter and the pRL-CMV Renilla internal control. Cells were treated with a vehicle, or increasing concentrations of DES (10pM to 1μM). Results are expressed as ± SEM (n=3). Statistical analysis was performed using one-way ANOVA, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3.4.3. Activation of an androgen response element in SK11 cells

As the cells were able to activate an oestrogen response element, a similar study was carried out using the pem-Luc androgen responsive promoter-reporter construct to investigate androgen responsiveness in the SK11 cells. Cells were co-transfected with pem-Luc and the internal control Renilla plasmid then stimulated with either testosterone or 17β oestradiol 4 hours after transfection to obtain a dose response. A luciferase assay was performed and the results were normalised and expressed as a fold increase compared to a no ligand control. A dose response curve was observed when the cells were stimulated with increasing concentrations of testosterone (10pM to 1μM) with maximum response of 2.5 fold being seen with 10nM (Figure 3.16A). The addition of E_2 did not result in activation of the androgen response element at any concentration of E_2 added (Figure 3.16B).

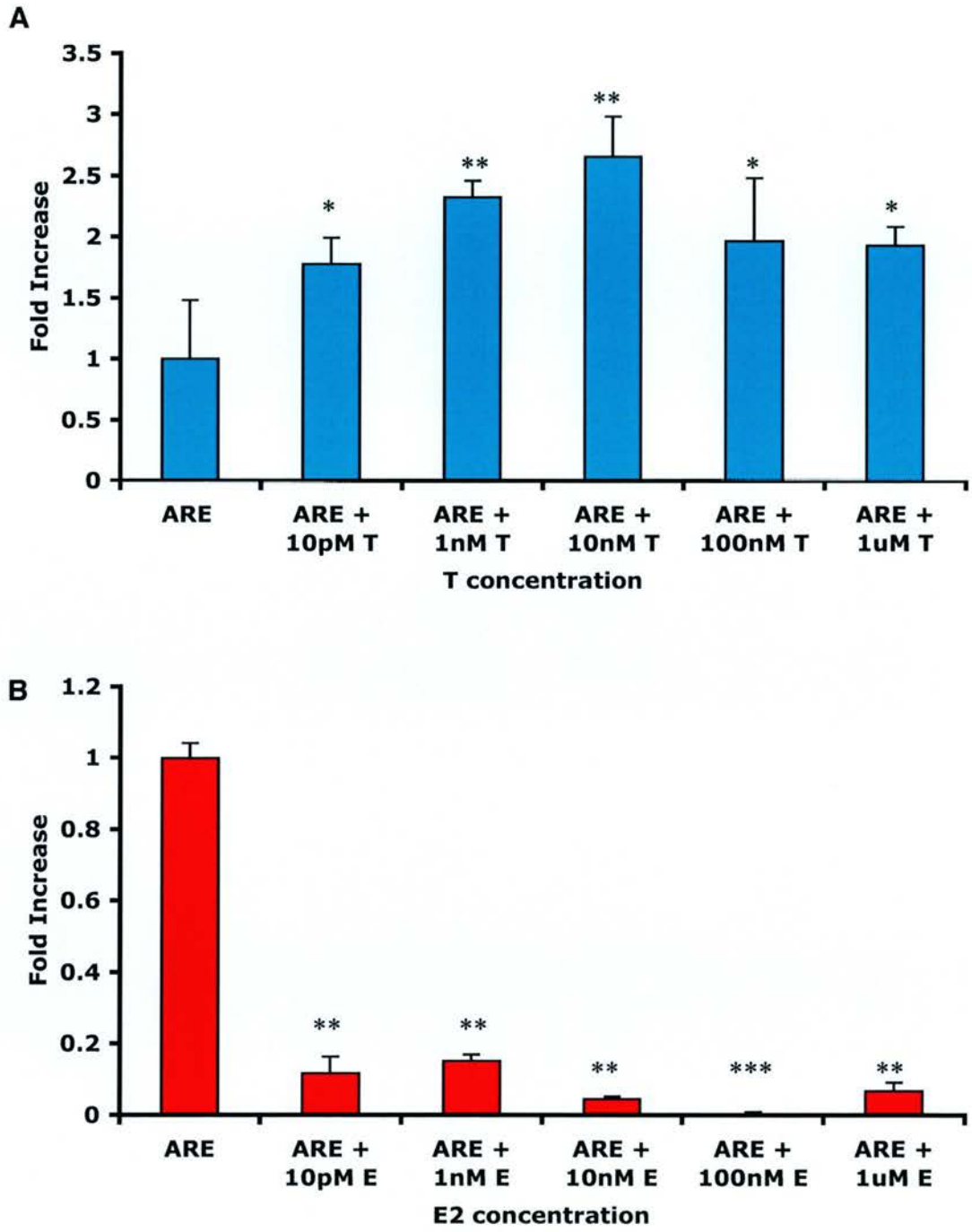


Figure 3.16 Androgen response in SK11 cells after ligand treatment. SK11 cells were transiently transfected with a pem-Luc reporter and the pRL-CMV Renilla internal control. Cells were treated with a vehicle, or increasing concentrations of (A) testosterone (10pM to 1μM) or (B) 17β oestradiol (10pM to 1μM). Results are expressed as ± SEM (n=3). Statistical analysis was performed using one-way ANOVA, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3.5. Sequence specific knockdown using RNAi

The ability to knockdown the expression of ERβ in the Sertoli cell is an exciting prospect, and this is one of the main aims of this chapter. RNA interference presents an opportunity to perform this but before it was carried out, the technique was optimised by experiments designed to knockdown various control genes to check that the use of this technique was a viable option.

3.3.5.1. Construction and transfection of control siRNA's

Control siRNA's for GFP, lamin, β-tubulin and β-actin were used to optimise conditions for siRNA transfection. An siRNA to GFP was constructed using the Silencer™ siRNA construction kit as detailed in section 2.12.2. Lamin A/C, α-tubulin and β-tubulin siRNA's were selected from the commercially available control siRNA sequences published by Dharmacon. These sequences have been shown to achieve high levels of knockdown in mammalian cells.

3.3.5.1.1. GFP

SK11 cells were transfected with a GFP expressing vector (pEGFP-C1, Clontech). Figure 3.18, panel A shows confocal images of the cells 48 hours after transfection with the GFP vector. Using an in-vitro transcribed siRNA to GFP, GFP expression in the cells was knocked down (Figure 3.17, panel B), shown by the absence of any green cells. Another siRNA, specific for the GAPDH gene was transfected along with GFP to show that non-specific knockdown was not occurring (Figure 3.17, panel C). Expression of GFP in these cells was unchanged showing that the reduction in GFP levels observed in the test samples was due sequence specific knockdown.

3.3.5.1.2. Lamin A/C

SK11 cells were transfected using a control siRNA for Lamin A/C supplied by Dharmacon. 48 hours after transfection, the cells were fixed and immunostained using an anti-lamin A/C antibody and observed using the confocal microscope (Figure 3.18, panel B). The cells were counterstained with propidium iodide. No

knockdown in lamin A/C expression in the SK11 cells was seen compared to untransfected cells (Figure 3.18, panel A).

3.3.5.1.3. α-tubulin

SK11 cells were transfected using a control siRNA for α-tubulin provided by Dharmacon. 48 hours after transfection, the cells were fixed and immunostained using an anti-α-tubulin antibody and observed using the confocal microscope (Figure 3.19, panel B). The cells were counterstained with To-Pro3. A reduction in the intensity of the α-tubulin staining in the SK11 cells was observed compared to untransfected cells (Figure 3.19, panel A).

3.3.5.1.4. β-tubulin

SK11 cells were transfected using a control siRNA for β-tubulin provided by Dharmacon. The cells were fixed and immunostained 48 hours after transfection using an anti-β-tubulin antibody and observed using the confocal microscope (Figure 3.20, panel B). The cells were counterstained with To-Pro3. High levels of knockdown in β-tubulin protein expression in the SK11 cells was seen compared to untransfected cells, shown in Figure 3.20, panel A.

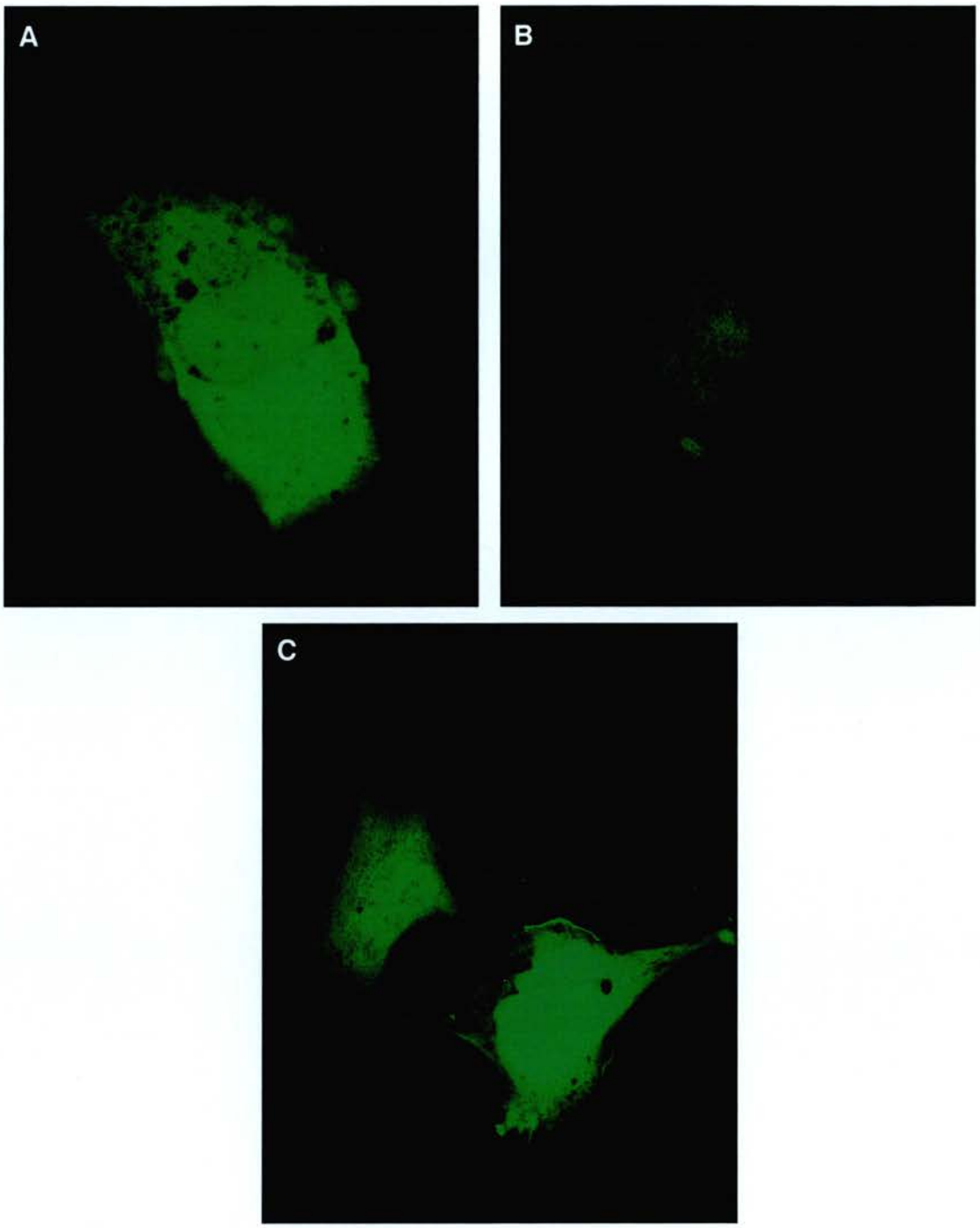


Figure 3.17 Knockdown of GFP expression in SK11 cells. Panel A shows cells transfected with a GFP vector. Panel B shows a reduction in the expression of GFP after co-transfection of GFP and siGFP. Panel C shows calls transfected with GFP and an siRNA to Gapdh.

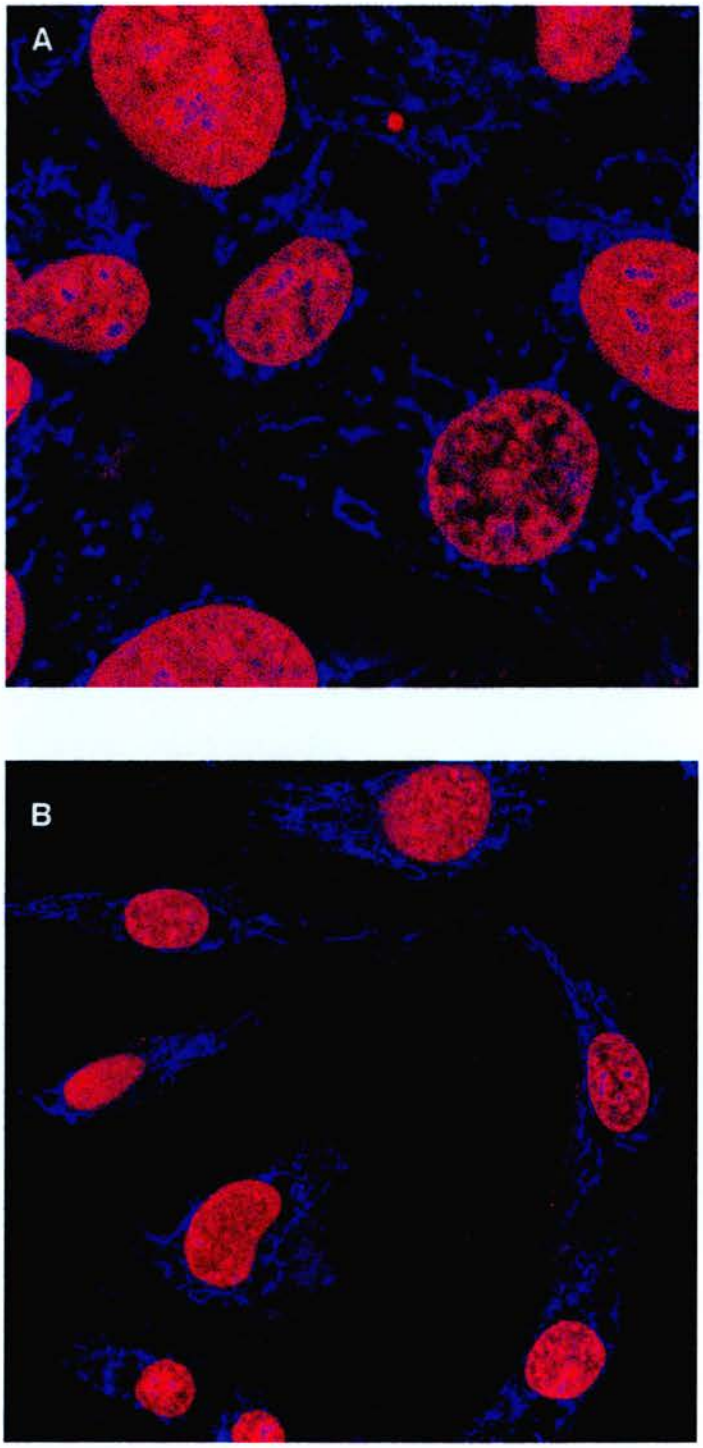


Figure 3.18 Panel A shows SK11 cells immunostained using an anti-lamin A/C antibody (blue) and counterstained with propidium iodide (red). Panel B shows SK11 cells transfected with a siRNA to lamin A/C prior to immunostaining with an anti-lamin A/C antibody (blue) and counterstained with propidium iodide (red).

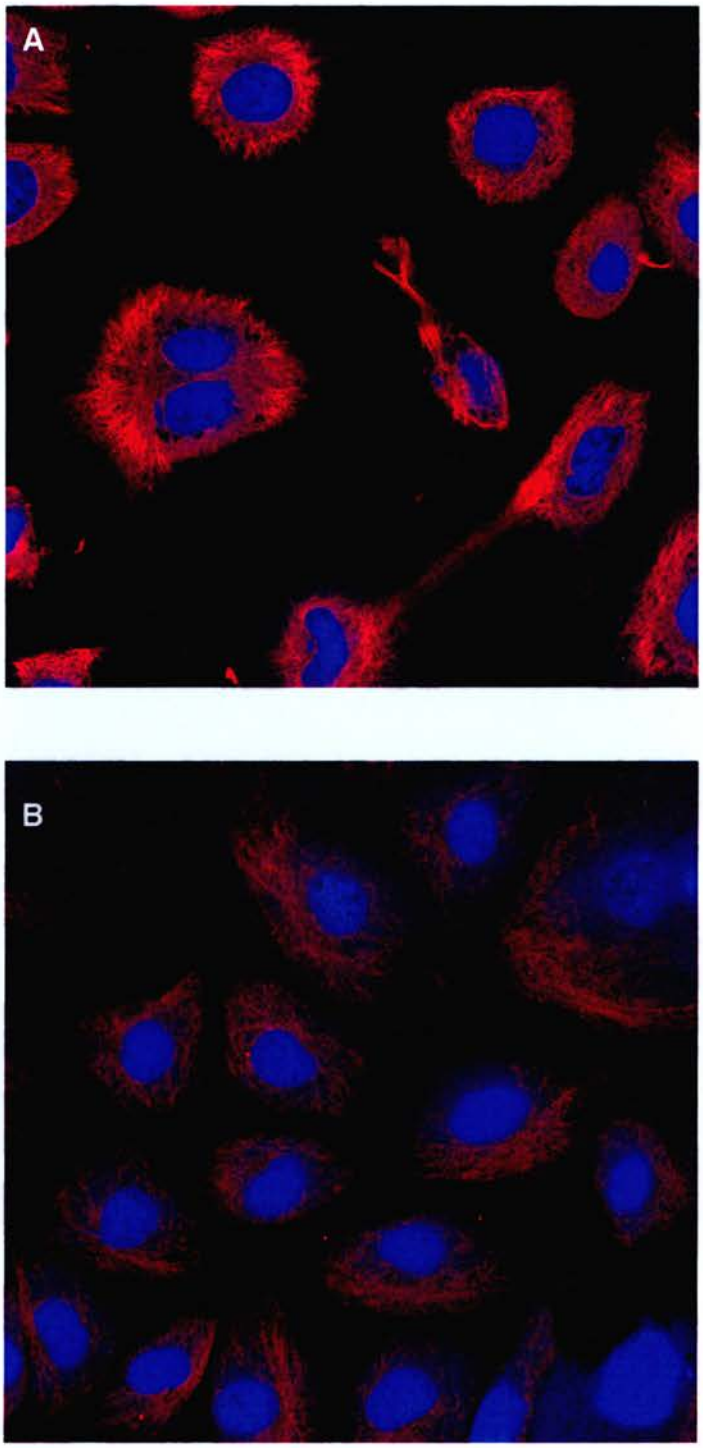


Figure 3.19 Panel A shows SK11 cells immunostained using an anti-α-tubulin antibody (red) and counterstained with To-Pro3 (blue). Panel B shows SK11 cells transfected with a siRNA to α-tubulin prior to immunostaining with an anti-α-tubulin antibody (red) and counterstained with To-Pro3 (blue)

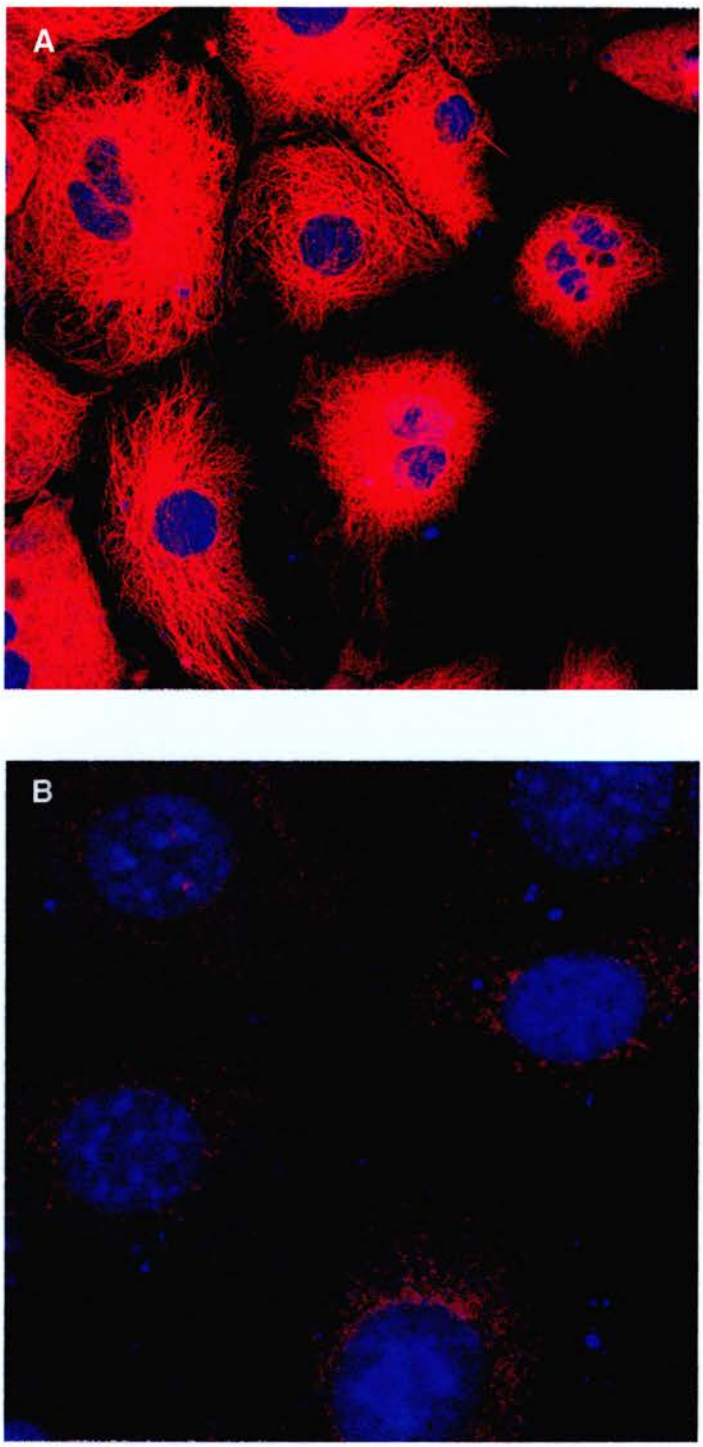


Figure 3.20 Panel A shows SK11 cells immunostained using an anti-β-tubulin antibody (red) and counterstained with To-Pro3 (blue). Panel B shows SK11 cells transfected with a siRNA to β-tubulin prior to immunostaining with an anti-β-tubulin antibody (red) and counterstained with To-Pro3 (blue)

3.3.6. siRNA labelling

In order to ascertain whether the siRNA's were able to enter the cells and to track the movement of the siRNA, two approaches were undertaken, the first involved labelling the siRNA with a fluorescent marker, this enabled visualisation of the siRNA after transfection. The second approach was to use a fluorescently labelled transfection reagent in order to track the movement of the siRNA into the cells.

3.3.6.1. Visualisation of the entry of fluorescently labelled siRNA's entry into SK11 cells

To check that the siRNA's were entering the cells, FAM labelled siRNAs for GAPDH (control sequence) and ERβ were transfected into cells and observed using a confocal microscope 24 and 48 hours after transfection. It was seen that after 24 hours, the siRNAs accumulated in the cytoplasm near the nucleus (Figure 3.21 A and C). By 48 hours post transfection the siRNAs appear in bright "spots" localized in the cytoplasm in close proximity to the nuclear membrane (Figure 3.21, B and D).

3.3.6.2. Visualisation of the movement of siRNA into SK11 cells using a fluorescently labelled transfection reagent.

Using a rhodamine labelled transfection reagent (Jet FluoR), siERβ was introduced into the SK11 cells. Figure 3.22A shows an accumulation of labelled transfection reagent carrying the siRNA around the nucleus of the SK11 cells 24 hours post transfection. Figure 3.22B shows cells transfected with the transfection reagent alone. No accumulation of fluorescent label is shown around the cells, which were counterstained with the nuclear stain To-Pro3.

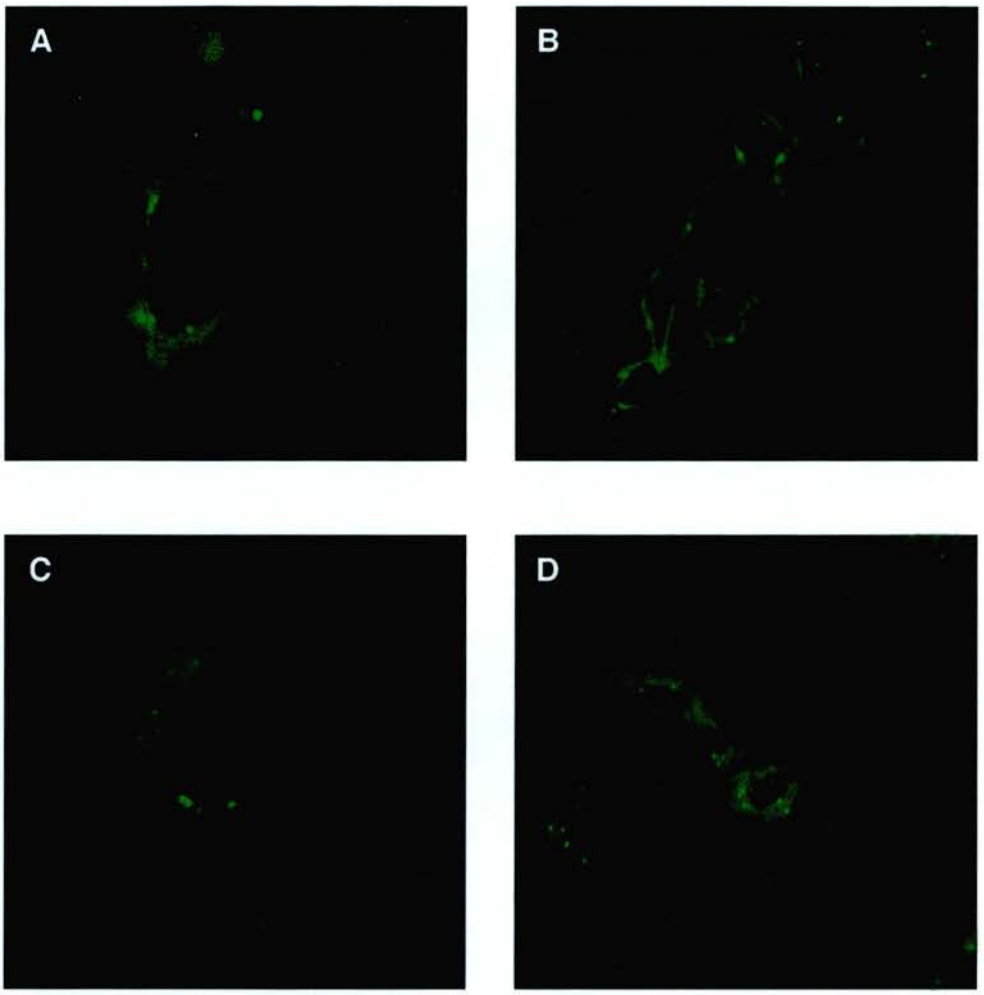


Figure 3.21 Panels A and B show SK11 cells transfected with FAM labelled siERβ. C and D show SK11 cells transfected with a control siGAPDH siRNA. Images were taken using a LSM 510 confocal microscope 24 (A and C) and 48 hours (B and D) after transfection.

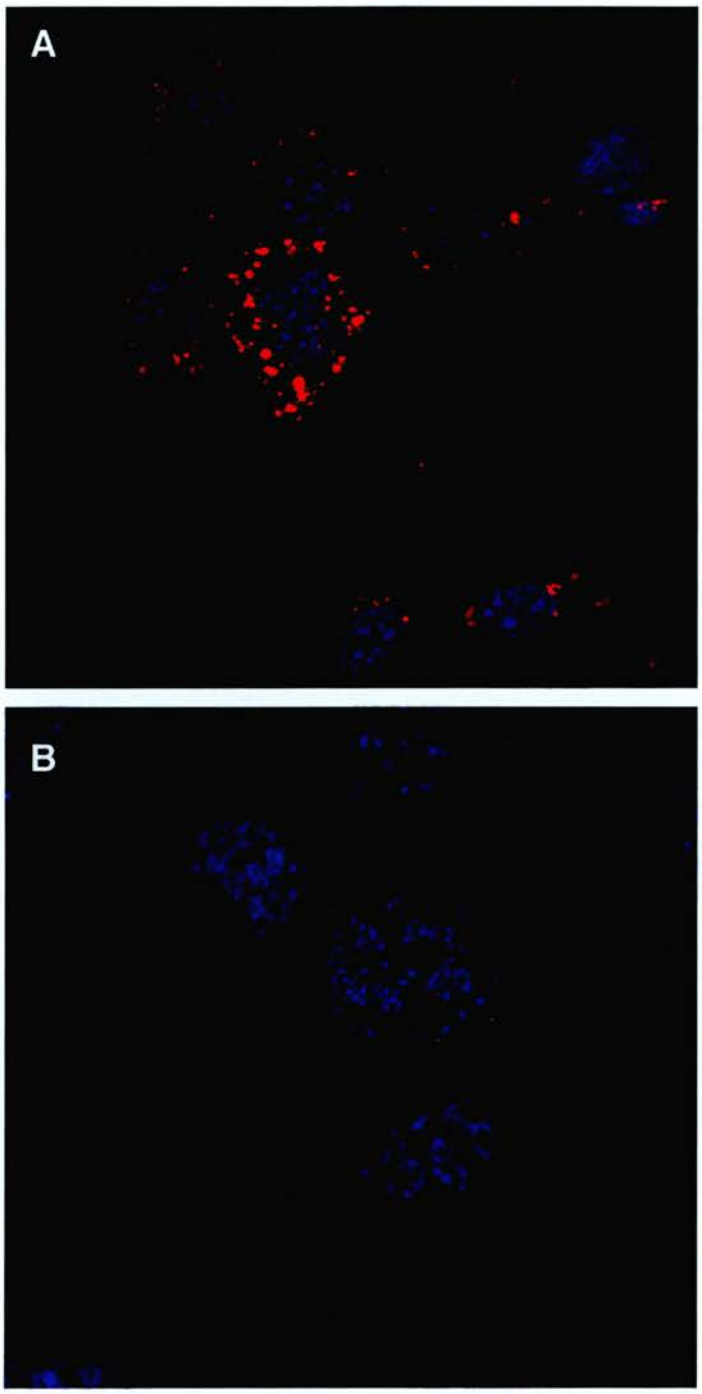


Figure 3.22 Panel A shows SK11 cells transfected with siERβ (red) using a rhodamine labelled transfection reagent and counterstained using to-pro 3 (blue). 48 hours after transfection, the siRNA has moved to the nuclear membrane of the cells. SiRNA. Panel B shows cell transfected with the transfection reagent only (no siRNA) and counterstained with To-Pro3 (blue).

3.3.7. Targeted knockdown of ERβ in SK11 cells

Once the technique had been shown to work effectively in the knockdown of control genes in the SK11 cells, it was decided to attempt to knock down the expression of ERβ within the SK11 cells.

A linear siRNA was chosen from the murine ERβ sequence as described in section 3.2.8.1. SK11 cells were transfected with ERβ-dsRed (section 3.2.7) and the siRNA to ERβ (section 3.2.8.4). 48 hours after transfection, the cells were visualised using the confocal microscope and the expression of ERβ protein was monitored using the expression of dsRed in the cells (Figure 3.23B). Cells transfected with ERβ-dsRed alone were used as a control (Figure 3.23A). The cells were counter-stained with To-Pro3 in order to visualise the nuclei of the cells. There was a reduction in the number of cells expressing dsRed in the cells transfected with ERβ-dsRed and siERβ compared to the cells transfected with ERβ-dsRed alone. Any cells treated with siERβ that were fluorescing under the confocal microscope showed a reduction in intensity of red fluorescence. Figure 3.23C shows cells transfected with ERβ-dsRed and siβ-tubulin. No reduction in the expression of ERβ-dsRed is seen in these cells showing that the knockdown observed in the cells is sequence specific.

Following the observation that the linear siRNA was effective at knocking down ERβ expression in the SK11 cells, a short hairpin RNAi (shRNA) was designed based on the linear siRNA sequence specific for ERβ and constructed in the pSilencer vector as described in section 2.12.6. Sequencing was performed to confirm the presence of the siERβ and the hairpin sequence and is shown in Figure 3.24A and B. The siERβ sequence is underlined and shown in red. The hairpin sequence is shown in blue and the Bam H1/Hind III sites shown in green with a portion of the pSilencer vector sequence surrounding the insert shown in black.

To test the efficiency of the shERβ SK11 cells were transfected with ERβ-dsRed and shERβ. Cells transfected with ERβ-dsRed alone were used as a control and all cells were counterstained with To-Pro3 to visualise cell nuclei. The cells were observed

on a confocal microscope 48 hours after transfection. In the cells transfected with shERβ (Figure 3.25B), no red cells were observed showing that knockdown of ERβ had been achieved in these cells using the hairpin vector. Figure 3.25, panel A shows cells transfected with ERβ-dsRed alone showing expression of dsRed in these cells.

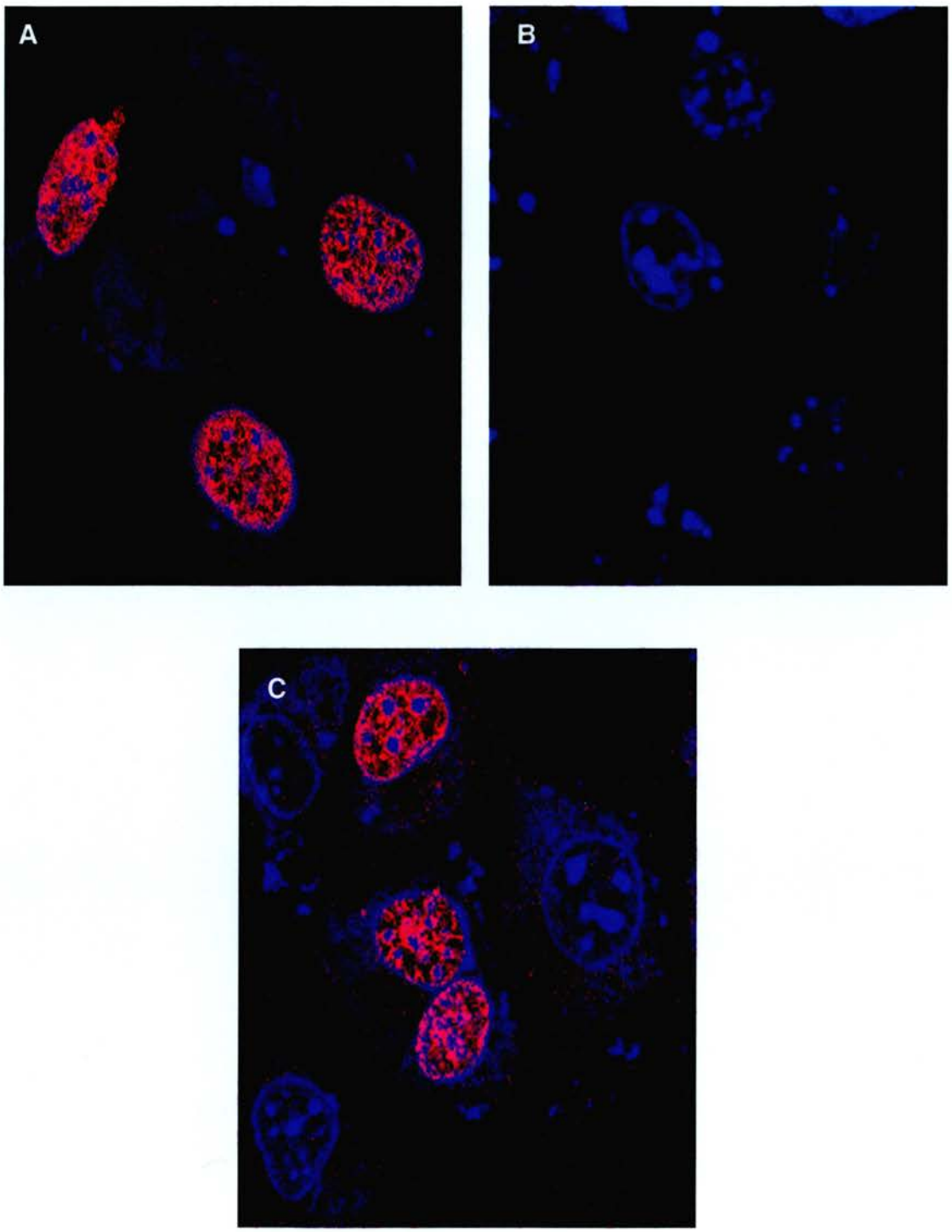


Figure 3.23 Confocal images showing knockdown of ERβ protein using a siRNA to ERβ. Panel A shows cells transfected with ERβ-dsRed. 48 hours after transfection. Panel B shows cells transfected with ERβ-dsRed and siERβ. Panel C shows SK11 cells transfected with ERβ-dsRed and a non-specific siRNA to β-tubulin. All cells were counterstained with To-Pro3 nuclear stain (blue).

A

CAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGC
TATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTT
GGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCA
GTGAATTCATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAAC
GTGAAATGTCTTTGGATTTGGGAATCTTATAAGTTCTGTATGAGACCACT
CGGATCCAAGAAGATAATGGTCAAGCTTCTCAAGAGAAAGCTTGACCAT
TATCTTCTTTTTTGGAAAAGCTTGGCGTAATCATGGTCATAGCTGTTTCC
TGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGA
AGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACAT
TAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTG

B

Figure 3.24 A shows the sequence of the shERβ hairpin containing pSilencer vector. The siERβ sense and antisense sequence are underlined and shown in red. The hairpin loop sequence is shown in blue with the BamH1/HindIII sites used to clone the hairpin insert shown in green. A portion of the pSilencer sequence at either side of the insert is shown in black. B shows how the hairpin folds up with the loop sequence shown in blue and the siERβ sense and antisense sequences shown in red.

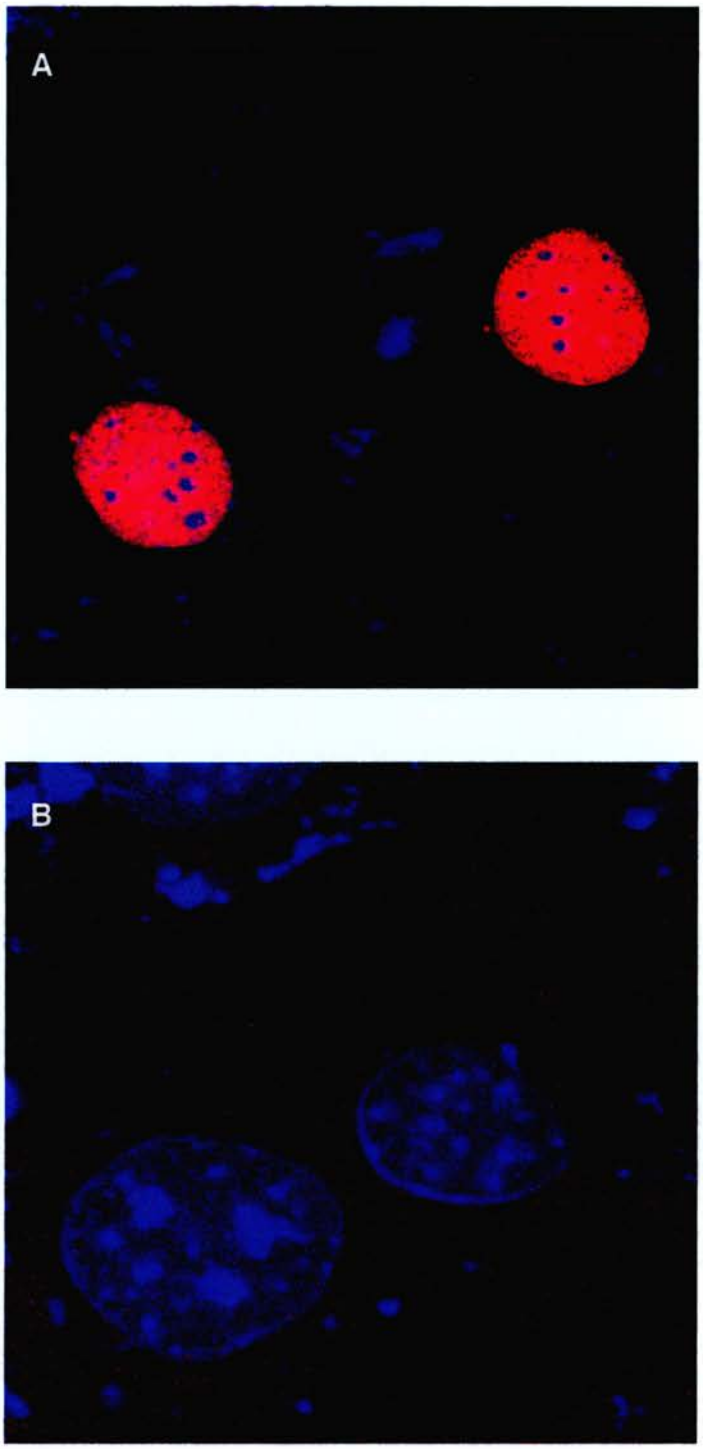


Figure 3.25 Knockdown of ERβ using a shRNA to ERβ. Panel A shows cells transfected with ERβ-dsRed. Panel B shows cells transfected with ERβ-dsRed (red) and shERβ. Images taken 48 hours after transfection using a LSM 510 confocal microscope. Cells were counterstained with To-Pro3 (blue).

3.3.7.1. Knockdown of ERE response using RNAi in SK11 cells

3.3.7.1.1. Linear siERβ

It has previously been shown in this chapter that the addition of 17β oestradiol can activate an ERE in the Sertoli cells after transient transfection (section 3.3.4.2). The ability of siRNA to knock down this response was investigated by co-transfection of a 3x ERE-luc construct (section 3.2.6) and the linear siRNA to ERβ followed by stimulation of the cells with increasing concentrations of 17β oestradiol (10pM to 1μM), luciferase activity was measured and fold increase in ERE response over control (no ligand added) was determined. Figure 3.26 (blue bars) shows SK11 cells transfected with ERE-Luc alone. A dose dependant increase in ERE activity is observed upon addition of E_2 with maximum response being seen at 100nM E_2. After transfection of siERβ alongside the ERE-Luc, no response to E_2 is observed at any concentration.

ERβ mRNA levels were investigated after transfection with ERE-Luc and siERβ by TaqMan quantitative RT-PCR. Figure 3.27 shows SK11 cells transfected with ERE alone (blue bars) with a dose dependant increase of ERβ mRNA expression after addition of E_2 at increasing concentrations (10pM to 1μM). Transfection of the cells with ERE-Luc and siERβ resulted in the knockdown of ERβ mRNA expression (Figure 3.27, pink bars).

3.3.7.1.2. shERβ

To establish whether the hairpin shERβ is as effective in knocking down ERβ expression in SK11 cells as the linear siRNA, the experiments were repeated using the shERβ vector. Activation of the 3x ERE-Luc construct with E_2 was shown to have been knocked down with the use of the shERβ vector (Figure 3.28). Luciferase activity was measured and fold increase in ERE response over control was determined. Blue bars show the response of the cells to 17β oestradiol (10pM to 1μM) after transfection with the 3x ERE-Luc, which gives a maximum response with

100nM E_2. This response is knocked down after transfection with the shERβ vector as shown by the pink bars. No response to E_2 is observed at any concentration.

Relative ERβ expression after transfection with the ERE-Luc construct and shERβ was measured by TaqMan RT-PCR and is shown in Figure 3.29 In agreement with the luciferase assay results, ERβ mRNA was reduced in the cells transfected with ERE-Luc and shERβ prior to stimulation with increasing concentrations of (10pM to 1μM) E_2 (pink bars) as compared to cells transfected with ERE-Luc followed by E2 stimulation with no shERβ added (blue bars).

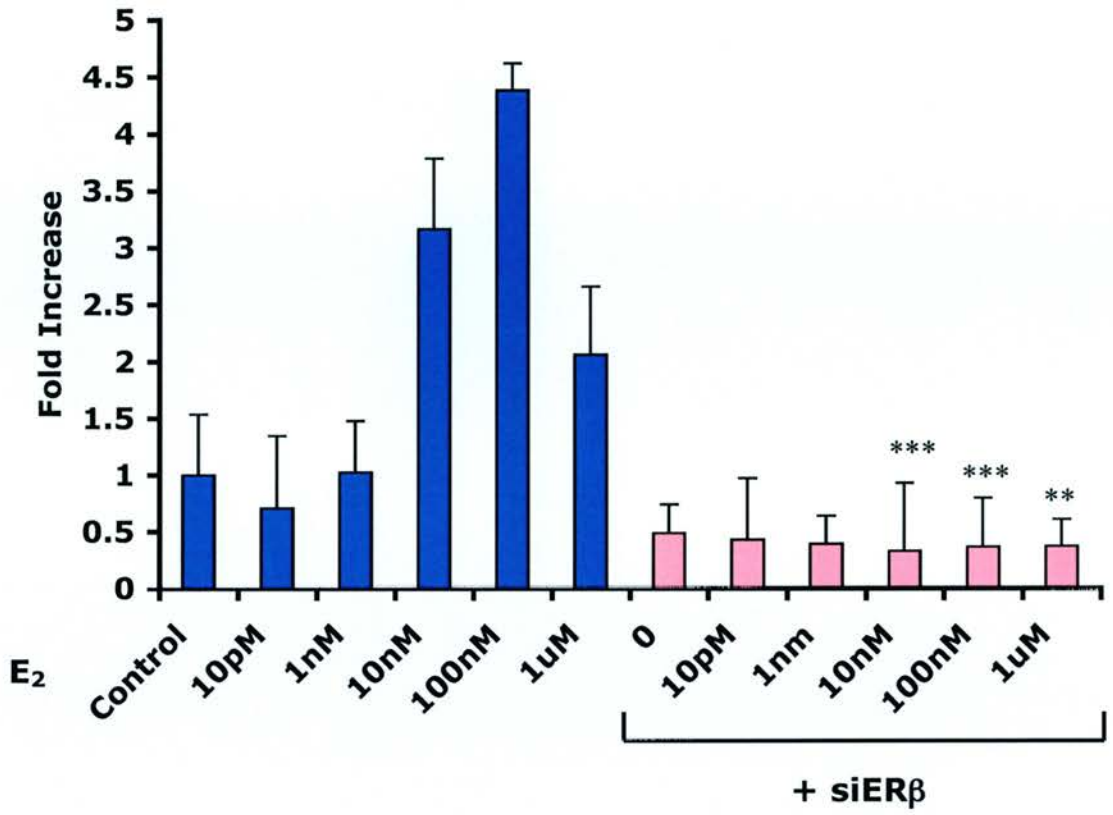


Figure 3.26 Knockdown of ERE response in SK11 cells after transfection with siERβ. The blue bars show the response of the SK11 cells transfected with a 3x ERE-Luc construct after stimulation with increasing concentration of 17β oestradiol (E_2). The pink bars show the response of the SK11 cells transfected with a 3x ERE-Luc construct and a siRNA to ERβ after stimulation with increasing concentration of 17β oestradiol. Results are ± SEM n=3. Statistical analysis to compare differences between samples treated with siRNA and those untreated was performed by the use of one-way ANOVA, ** $P < 0.01$; *** $P < 0.001$.

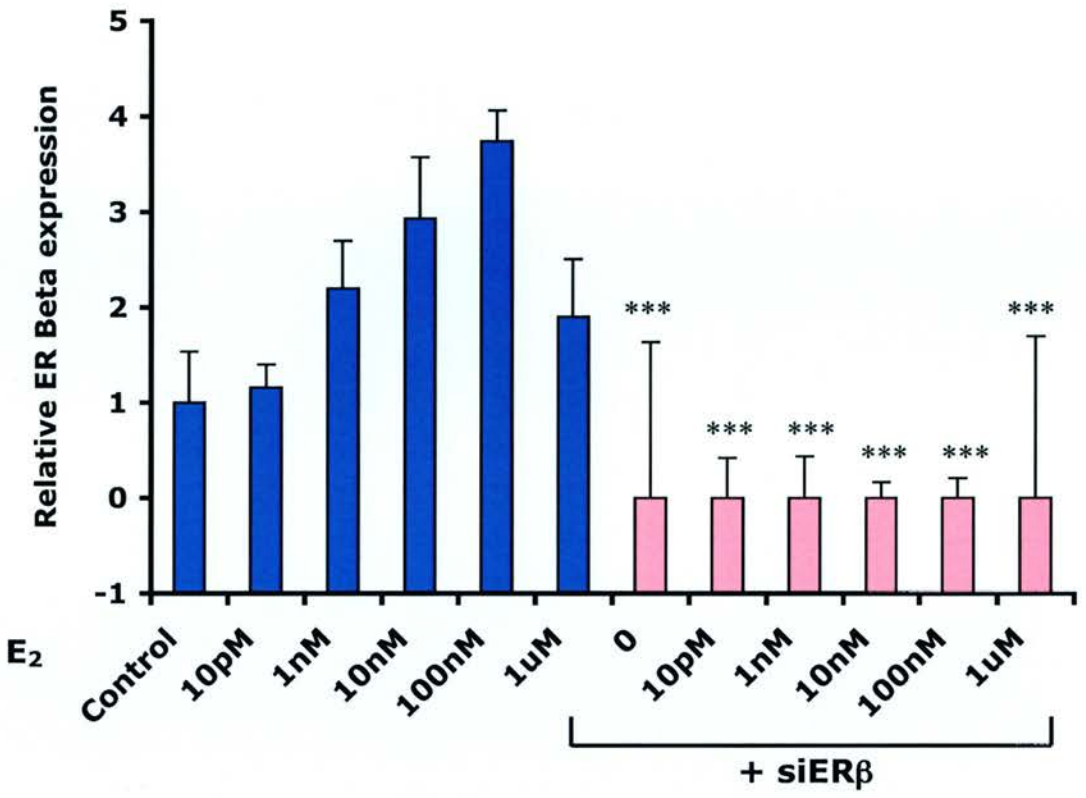


Figure 3.27 Effect on ERβ mRNA expression after transfection with a 3xERE-Luc reporter construct and siERβ. Blue bars show an increase in ERβ mRNA expression after stimulation with increasing concentrations of E_2. The pink bars show a reduction in ERβ mRNA levels after transfection of ERE-Luc and siERβ followed by stimulation with increasing concentrations of E_2. Results are ± SEM n=3. Statistical analysis to compare differences between samples treated with siRNA and those untreated was performed by the use of one-way ANOVA, *** $P < 0.001$.

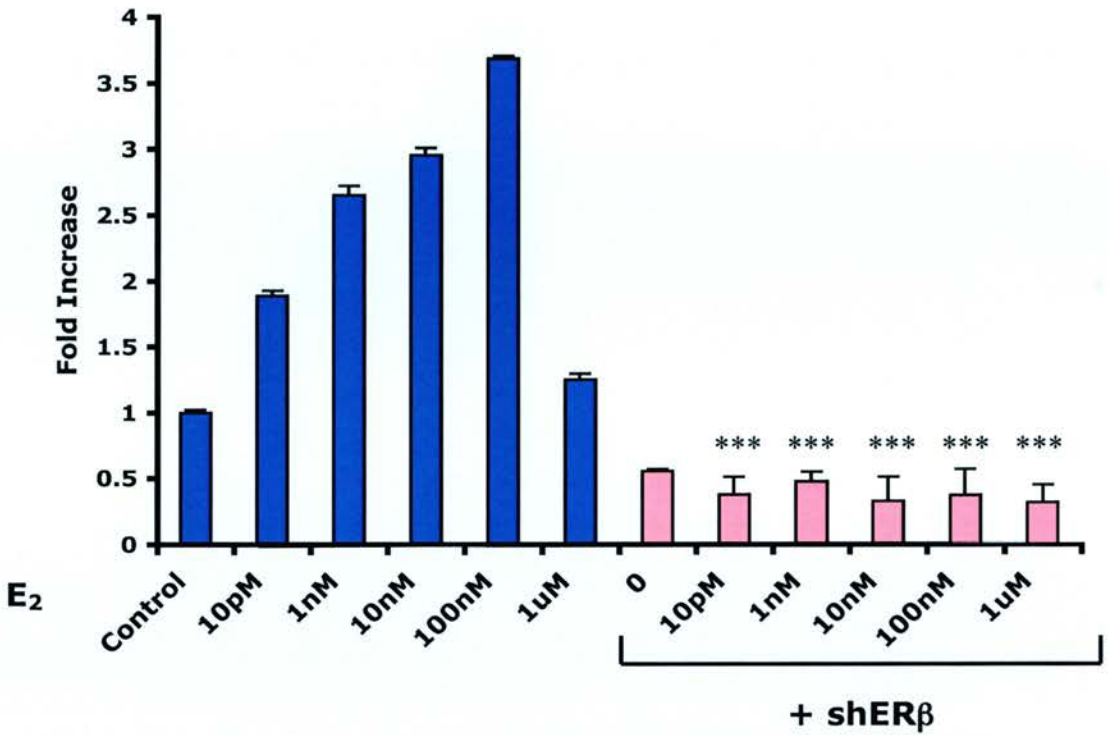


Figure 3.28 Knockdown of ERE response in SK11 cells after transfection with shERβ. The blue bars show the response of the SK11 cells transfected with a 3x ERE-Luc construct after treatment with increasing concentrations of E_2. The pink bars show the response of the SK11 cells transfected with a 3x ERE-Luc construct and an shRNA to ERβ after stimulation with increasing concentration of E_2. Results are ± SEM n=3. Statistical analysis to compare differences between samples treated with siRNA and those untreated was performed by the use of one-way ANOVA, *** $P < 0.001$.

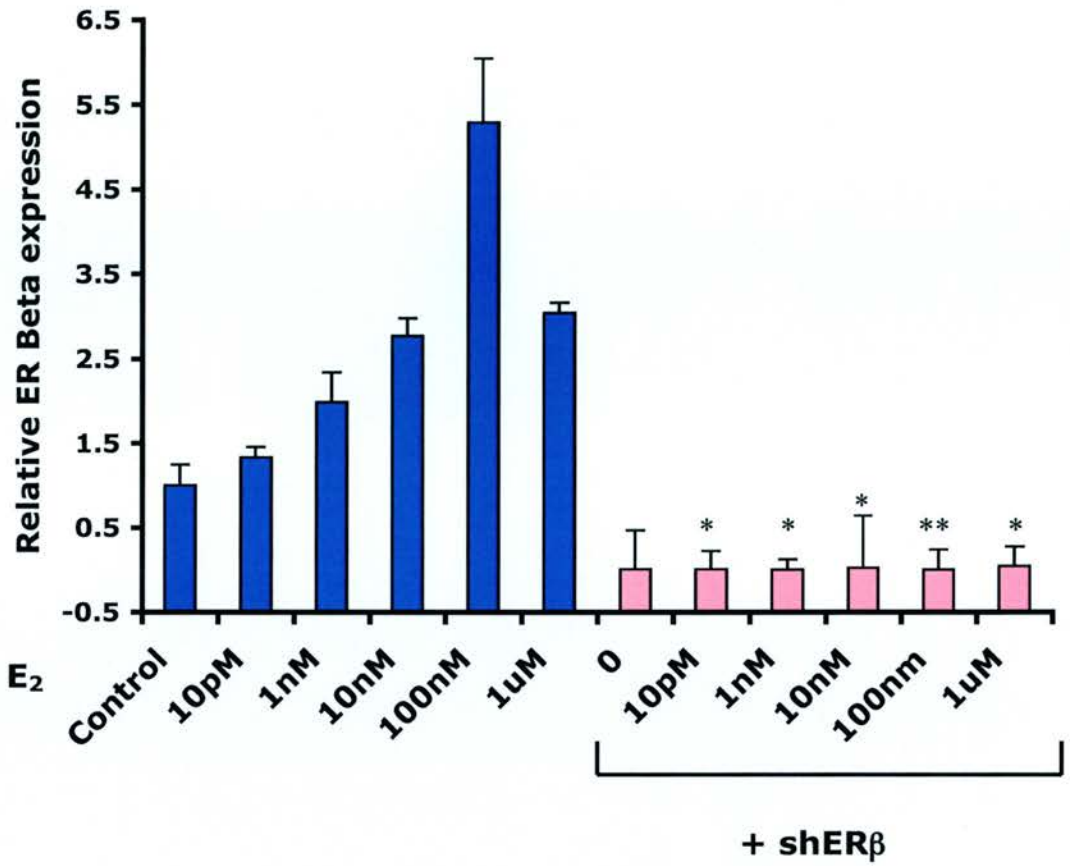


Figure 3.29 Knockdown of ERE response to E2 in SK11 cells. Relative ERβ mRNA expression in reduced (pink bars) when SK11 cells are transfected with a 3x ERE-Luc construct and shERβ then stimulated with increasing concentrations of E2 compared to cells transfected with ERE alone prior to stimulation with E2 (blue bars). Results are ± SEM, n=3. Statistical analysis to compare differences between samples treated with siRNA and those untreated was performed by the use of one-way ANOVA, * $P < 0.05$; ** $P < 0.01$.

3.3.7.2. Knockdown of ERβ mRNA in SK11 cells using RNAi

The previous section (3.3.7.1) has shown that the cells response to oestrogenic stimulation after transfection with an ERE-reporter construct can be blunted with the use of RNA interference. The following experiments were performed to investigate whether endogenous ERβ levels within the SK11 cells could be knocked down using RNAi, after oestrogenic stimulation.

3.3.7.2.1. siERβ

Levels of ERβ (without transfection of the ERE reported construct) after knockdown using the linear siRNA to ERβ were investigated using TaqMan quantitative RT-PCR. Cells were transfected with siERβ and stimulated with increasing concentrations of E_2 (10pM to 1μM) 48 hours later, RNA and cDNA was prepared from the cells for Taqman analysis. ERβ mRNA levels were compared to cells not transfected with siERβ. ERβ mRNA levels after stimulation with E_2 is shown in Figure 3.30 (blue bars). Unstimulated cells were used as a control. A dose dependant increase of ERβ mRNA is seen. This response is knocked down after transfection with siRNA (Figure 3.30, pink bars).

3.3.7.2.2. shERβ

Relative ERβ amounts were determined in SK11 cells after transfection with the shERβ vector and stimulation with E_2 by Taqman quantitative RT-PCR. Cells, both transfected and untransfected with the shERβ vector were stimulated with 10pM to 1μM E_2 for 48 hours. Unstimulated cells were used as a control. Again, a dose dependant increase of ERβ mRNA is seen after stimulation with E_2 (Figure 3.31, blue bars) and this response is knocked down after transfection with the shERβ vector (Figure 3.31, pink bars)

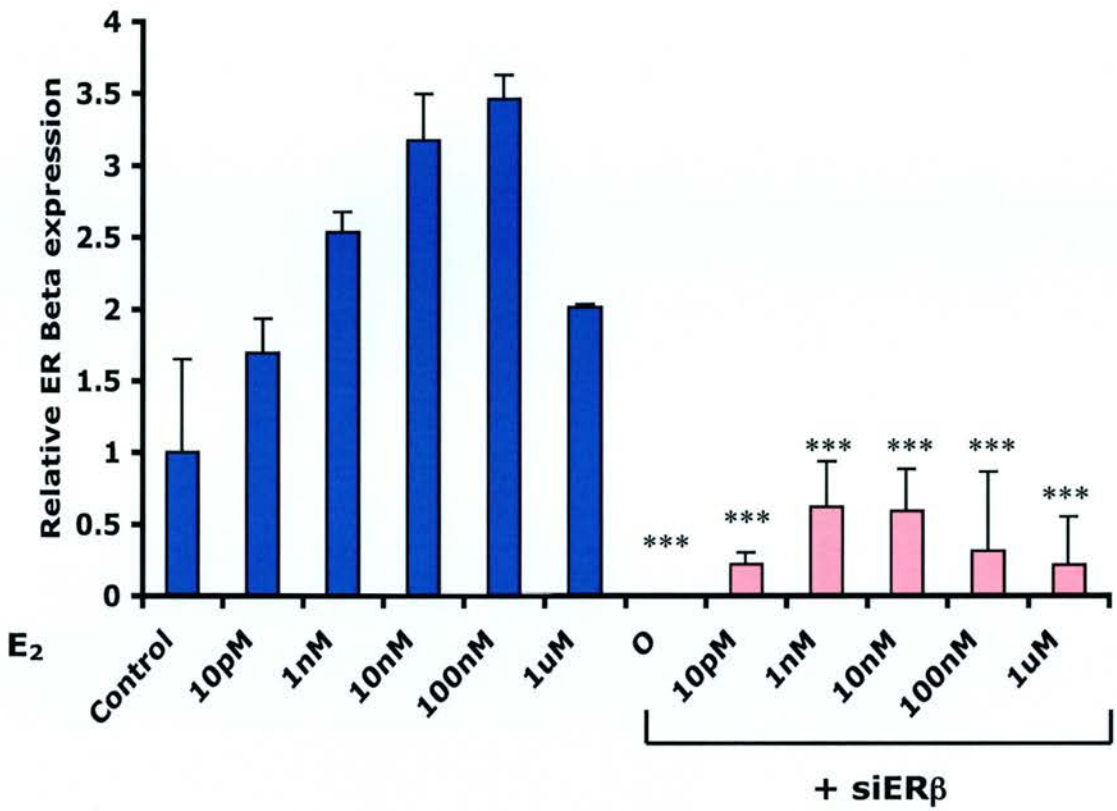


Figure 3.30 Reduction in ERβ mRNA levels in SK11 cells after transfection with siERβ. Blue bars show relative ERβ mRNA expression in cells stimulated for 48 hours with increasing concentrations of 17β oestradiol (E_2). Pink bars show relative ERβ mRNA expression in cells transfected with siERβ then stimulated with increasing concentrations of 17β oestradiol (E_2) for 48 hours. Results are ± SEM n=3. Statistical analysis to compare differences between samples treated with siRNA and those untreated was performed by the use of one-way ANOVA, *** $P < 0.001$.

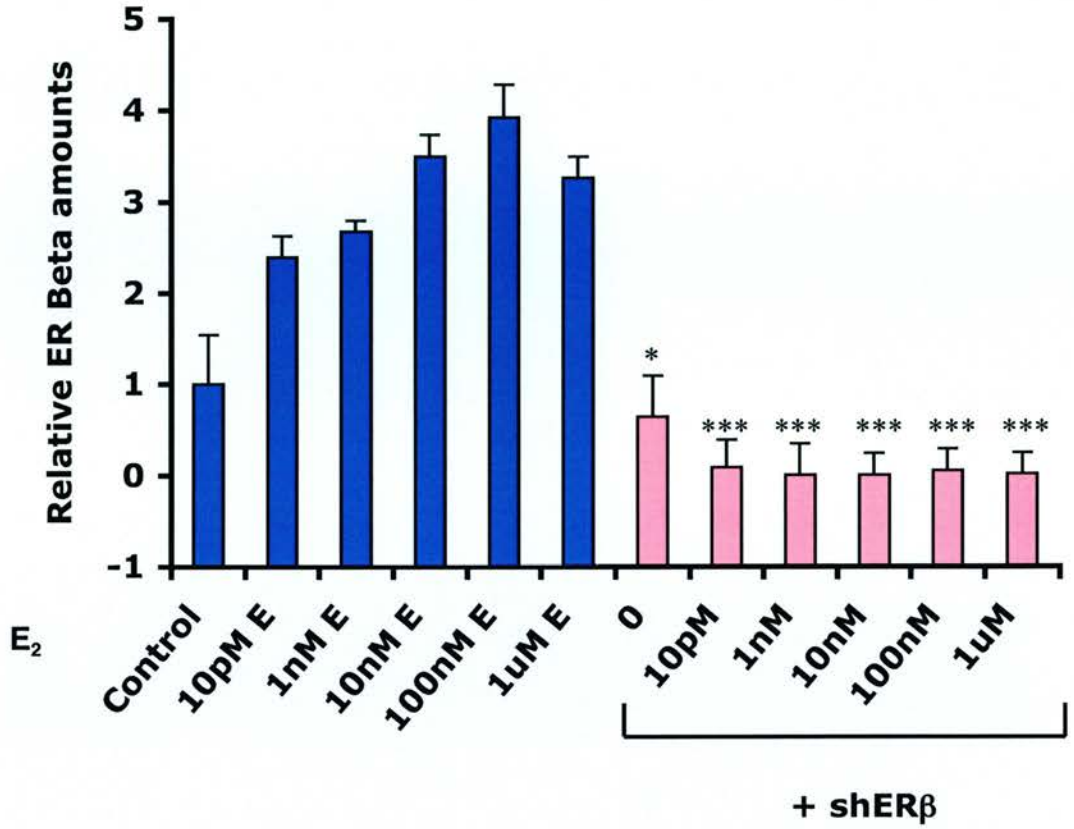


Figure 3.31 Reduction in ERβ mRNA levels in SK11 cells after transfection with shERβ. Blue bars show relative ERβ mRNA expression in cells stimulated for 48 hours with increasing concentrations of 17β oestradiol (E_2). Pink bars show relative ERβ mRNA expression in cells transfected with shERβ then stimulated with increasing concentrations of 17β oestradiol (E_2) for 48 hours. Results are ± SEM n=3. Statistical analysis to compare differences between samples treated with siRNA and those untreated was performed by the use of one-way ANOVA, *** $P < 0.001$.

3.4. Discussion

In order to fully understand the role of the Sertoli cell in spermatogenesis, several attempts have been made to create Sertoli cell lines. The current study has used one of these, the SK11 cell line (Walther et al., 1996). Other cell lines, including the one described by Hofmann et al have been shown to express various Sertoli cell products including SCF, SGP-2 and GATA-1 (Hofmann et al., 2003). However, this cell line does not express androgen receptor or FSH receptor and as a result, this line is not suitable for carrying out studies investigating the hormonal control of spermatogenesis. The cell line TM4 (Mather, 1980) was derived from continued passaging of testicular cells and is presumed to be of Sertoli cell origin. This cell line does not show a differentiated phenotype and is therefore not ideal for studying differentiated Sertoli cell function. Another cell line has been produced by transfecting in expression vectors containing the FSH receptor in an attempt to get the cells to express the receptor resulted in cells that were responsive to hormonal stimulation but they do not proliferate *in vitro* and the cells do not behave as Sertoli cells *in vivo* (Eskola et al., 1998). Although no cell line will ever recreate the *in vivo* situation exactly, looking at this evidence and the many other Sertoli lines described in the literature, it seems apparent that the SK11 cell line shares many features exhibited by Sertoli cells *in vivo* and is therefore a valuable tool in the study of Sertoli cell function.

One of the main features of the SK11 cells is that they can be maintained in both an undifferentiated and differentiated state. After differentiation of the cells, which occurs after inactivation of the SV40 large-T antigen (Walther et al., 1996), a reduction in growth is observed and a morphological change in cell shape is seen. This change in morphology was demonstrated after immunostaining for β-actin protein in the undifferentiated and differentiated cells, a change in the cytoskeletal architecture was observed with an increased accumulation of β-actin protein around the nucleus of the differentiated cells. This is accompanied with an increase in β-actin mRNA expression in the differentiated SK11 cells.

This chapter investigated the expression profile of the Sertoli cell line SK11 and compared the expression of various Sertoli cell products in the cell line with Sertoli cells within the intact mouse testis using RT-PCR and immunohistochemistry. Sulphated glycoprotein (SGP-1), which is a secretory product of Sertoli cells (Collard and Griswold, 1987) mRNA and protein was localised to the cytoplasm of the mouse testis and in the SK11 cells, both differentiated and undifferentiated. Androgen receptor, another well known Sertoli cell product was localised to the nuclei of the SK11 cells and to Sertoli cell nuclei of fixed sections of mouse testis and was shown to be expressed in higher levels in the differentiated cells. Androgen receptor was also expressed in the Leydig cells and peritubular myoid cells of the testis but expression was absent from the germ cells. This expression pattern is consistent with previous studies that report expression of these genes in the Sertoli cells of the mouse (Walther et al., 1996) and the results obtained in this study show that the SK11 cells exhibit a molecular phenotype similar to that of Sertoli cells *in vivo* even after being in culture for a prolonged period of time.

The expression of ERβ and AR has been demonstrated in the SK11 cells in this chapter. ERβ is expressed in multiple cell types including Sertoli cells, in the d10 and adult testis and in undifferentiated and differentiated SK11 cells. As previously mentioned, AR expression in Sertoli cells is induced postnatally in the mouse. It was observed in these studies that the differentiated SK11 cells expressed higher levels of AR protein and mRNA compared with those maintained at 33°C (undifferentiated), which fits with the observation that AR expression in the mouse testis increases after Sertoli cell differentiation.

The fact that the SK11 cells express steroid hormone receptors suggested that they would be responsive to oestrogens and androgens. This was demonstrated when the cells were hormonally stimulated with either 17β oestradiol or testosterone. This stimulation resulted in an increase in relative expression of both receptors as determined by real time PCR. This was further tested by carrying out transient transfections using both an oestrogen response element reporter construct and an

androgen response element containing promoter construct. The results show that upon addition of oestrogenic ligands, transcriptional activation of the ERE was achieved. Previous studies using the human ER have indicated that different ligands cause differing conformational changes in the receptor upon binding to the response element resulting in varying levels of transcriptional activation (Hall et al., 2002). In this study, six different oestrogenic ligands were added to the cells after they had been transfected with an ERE reporter construct to investigate if the endogenous ER in the SK11 cell line was functional. The results obtained showed that treatment with 100nM 17β oestradiol resulted in receptor-induced activation of transcription. This effect was also demonstrated when the ligands DES and DPN were used. After addition of genistein and 3β Adiol, an increase in transcription was obtained at a ligand concentration of 1μM. These results are consistent with the expression of ERβ protein within the Sertoli cells.

Various studies have reported that ERα is not expressed in Sertoli cells in various species (Saunders et al., 2001; Zhou et al., 2002). This study has shown the SK11 cell line does not express ERα mRNA or protein as demonstrated by RT-PCR and immunohistochemistry. Further proof of the absence of the receptor was given when the cells were transfected with the ERE reporter construct and stimulated with PPT™. The results in this study have shown that this ERα specific ligand did not activate gene transcription via the ERE upon transient transfection.

Cells were also transfected with an ARE containing reporter construct and stimulated with testosterone; this induced an increase in expression of the reporter showing that the cells contained a functional AR. No response was observed when the cells were transfected and stimulated with E_2. The levels of testosterone-induced response seen after transfection were lower than expected, as these were preliminary results, further work is required to optimise conditions for transfection. As it has been shown that levels of androgen receptor increase in differentiated SK11 cells, it would be interesting to see if higher levels of transcriptional activation after androgen stimulation could be achieved in the cells after culture at 39°. Future work

investigating the response of the SK11 cells using the androgen and oestrogen response elements may help in understanding of the role of the androgen and oestrogen receptors in the Sertoli cell and this in turn, may reveal information of the relationship between androgens and oestrogens in the testis.

According to most studies, and the data presented in this chapter, male germ cells are devoid of the androgen receptor (Bremner et al., 1994; Zhou et al., 2002), it is clear however from evidence in the literature that androgens acting on Sertoli cells are essential for efficient spermatogenesis.

Androgens and AR are required for normal spermatogenesis, yet AR is not expressed in all cell types of the testis. Evidence for roles of AR in spermatogenesis have been shown through the study of AR-deficient mice (O'Shaughnessy et al., 2002; Yeh et al., 2002), AR-deficient mice, both Tfm and ARKO, are infertile and display feminized internal and external phenotypes but due to the developmental roles of AR, removal of AR in the whole animal does not allow the study of AR function in specific cell types in the testis (de Krester and Kerr, 1994). Recent data involving the development of a Sertoli cell specific androgen receptor knockout mouse (SCARKO) gave proof that androgens are likely to be having an effect on spermatogenesis indirectly via the Sertoli cells as removal of the receptor resulted in infertility associated with a reduction in the numbers of spermatocytes and spermatids causing impaired spermatogenesis (De Gendt et al., 2004). Soon after this report, another conditional Sertoli cell androgen receptor knockout mouse was described, also exhibiting infertility due to a spermatogenic arrest at the premeiotic diplotene spermatocyte stage (Chang et al., 2004). These mice models demonstrate that the AR in Sertoli cells is essential for the maintenance of fertility. As the Sertoli cells express both AR and ERβ, interactions between the two receptors may be a major regulatory force in the control of spermatogenesis but the data from the conditional Sertoli cell knockouts clearly shows that even although ERβ is co-expressed with AR in Sertoli cells, it is not able to compensate for the loss of AR in the SCARKO mice and the role of ERβ in the Sertoli cell remains unknown.

The information gained from knockout animals can be very valuable and with the advent of conditional knockout technologies this has become even more exciting. However, the production of such animals is time consuming and expensive and it was with this in mind that studies using RNA interference was carried out in this thesis. As described in chapter one, RNAi presents an opportunity to knockdown gene expression in cells and tissues. In this study, the expression of ERβ and AR was attempted in the SK11 cells. Initial optimisation of the technique was carried out using control sequences, which were claimed to give good knockdown in mammalian cell lines. Using a sequence for GFP, knockdown of a transfected GFP vector in the SK11 cells was seen by confocal microscopy. This was followed by attempts to knockdown the cytoskeletal proteins α-tubulin, β tubulin and lamin A/C. Out of these three siRNA's, only that directed against β-tubulin showed a good level of knockdown. This is an important observation, as it shows how published sequences supposedly guaranteed to give knockdown do not always work. This could be due to the cell lines or the method of staining used.

Visualisation of the siRNA moving into the cells using fluorescently labelled siRNA's and transfection reagents was performed. These techniques allow the intracellular delivery and trafficking of siRNA's and can be useful in determining if any lack of knockdown observed is due to the siRNA not entering the cell. In the case of GFP, GAPDH and ERβ, the movement of the siRNA was observed over a period of time and the siRNA was seen to enter the cytoplasm and take up position surrounding the nucleus of the cells. Once the technique was optimised, knockdown of ERβ and AR in the SK11 cells was attempted. An siRNA to ERβ was chosen and knockdown of ERβ protein and mRNA was achieved in the SK11 cells. Using this sequence, a short hairpin RNAi was constructed in the pSilencer vector, this was seen to give slightly better levels of knockdown of ERβ protein expression as shown by confocal microscopy using a fluorescently tagged ERβ transfected into the cells. The ability of the cells to respond to E_2 and induce expression of a 3x ERE reporter construct after transfection with both the siERβ and shERβ constructs was also investigated and knockdown of E_2 induced transcriptional activation was achieved.

These experiments showed that the induction of reporter gene expression was blunted consistent with knockdown of endogenous ERβ protein levels.

Little progress was made in achieving knockdown of androgen receptor mRNA in SK11 cells. The design of an effective siRNA sequence can be "hit or miss" and in this case, none of the sequences used showed any effect on the AR levels within the cells. Further work will be required to design a sequence that is capable knocking down endogenous AR in Sertoli cells.

One of the advantages of using plasmid delivered siRNA is that longer-term knockdown can be achieved with the levels of mRNA and protein remaining reduced for up to 12 days. This effect would be advantageous if the technique was transferred to *in-vivo* studies and used for direct injection of shRNA vectors into the testis. It has been shown that adenoviruses are preferentially taken up by Sertoli cells (Scobey et al., 2001). If an shRNA was cloned into an adenoviral vector then introduced into the testis, the effects of knocking down AR/ERβ specifically in the Sertoli cells could be observed and it could be determined whether results are similar to that seen in the SCARKO animals. These studies are planned.

The studies performed in this chapter have shown that the SK11 cell line is an excellent system to study the effects of androgens and oestrogens on Sertoli cells and to try and understand the role of steroid hormone receptors in the seminiferous epithelium. The optimisation of RNA interference technology in these cells has been an important breakthrough in the use of this method for further understanding of the process of spermatogenesis and further studies using this method should yield very interesting results.

Chapter 4

Isolation, characterisation and in vitro differentiation of murine spermatogonial stem cells

4.1. Introduction

In the adult testis, spermatogonial stem cells are located on the basement membrane of the seminiferous tubule. These cells divide to populate the testis with germ cells that are committed either to a differentiation pathway or to maintain the stem cell population (de Rooij, 2001; de Rooij and Grootegoed, 1998; Kent Hamra et al., 2004; McLean et al., 2003; Oakberg, 1971). Spermatogonial stem cells and germ cell development have been extensively studied in mice and a wide range of transgenic mice exist with defects in spermatogenesis resulting in infertility or subfertility (Cooke and Saunders, 2002; de Rooij and de Boer, 2003; Matzuk and Lamb, 2002). These highlight the usefulness of using the mouse as a model for the understanding of germ cell development.

4.1.1. Development of spermatogonia

Stem cells are the only cells that are capable of sustained self renewal (de Rooij and Russell, 2000). Spermatogonial stem cells originate from primordial germ cells (PGCs). By embryonic day 7 of mouse development, about 100 PGCs can be detected in the extra-embryonic mesoderm, which lies posterior to the primitive streak (Ginsburg et al., 1990). The PGCs migrate along the hindgut into the genital ridges. The cells proliferate during migration and by the time they reach the genital ridges, which is around embryonic day 13 in the mouse, they have increased in number to about 10,000 cells in each gonad (Tam and Snow, 1981). The PCGs are arrested at the G0/G1 stage at around day embryonic day 16.5. Once in the genital ridges, the PGCs are in close contact with the Sertoli cells and become enclosed in the seminiferous cords, where they are termed gonocytes (Huckins and Clermont, 1968). Differences exist between PGCs and gonocytes. For example, gonocytes are only able to survive in the presence of Sertoli cells but PGCs can be successfully cultured with other cell types suggesting that gonocytes have a restricted

differentiation potential (Resnick et al., 1992; van Dissel-Emiliani et al., 1993). The mitotically quiescent gonocytes, re-enter the cell cycle shortly before birth and by half a day after birth, gonocytes are uniformly round and separated from the basement membrane (Clermont and Perey, 1957; Huckins and Clermont, 1968; Vergouwen et al., 1991). The gonocytes then migrate to the basement membrane and spermatogenesis begins with the differentiation of the cells to type A spermatogonia (Bellve et al., 1977; Huckins and Clermont, 1968). This movement is thought to be critical to ensure survival of these cells, as it has been observed that gonocytes remaining in the centre of testis cords eventually degenerate (Hasthorpe et al., 1999). It has been suggested by de Rooij (1998) that the gonocytes are a heterogeneous population in which only some of cells retain stem cell properties while the others are destined to enter the differentiation pathway during spermatogenesis (de Rooij and Grootegoed, 1998). Figure 4.1 summarises the pattern of development that leads to the formation of spermatogonia.

Stem cells exist in a specialized cellular compartment termed a "niche" that promotes stem cell survival/maintenance and excludes factors that induce differentiation (Schofield, 1978). The presence of such a niche in the testis was discovered during the development of the germ cell transplantation technique (Shinohara et al., 2001). After transplantation, stem cells migrate down into the tubule, passing through Sertoli cell tight junctions to colonise sites on the basement membrane of the tubule (Spradling et al., 2001). When a stem cell divides in its niche, only one daughter cell can remain, the other cell will be committed to differentiate (Schofield, 1978). The spermatogonial stem cell niche is defined in part by close associations with Sertoli cells which line the basement membrane of the seminiferous tubules (Orth et al., 2000; Ryu et al., 2003; Shinohara et al., 2001).

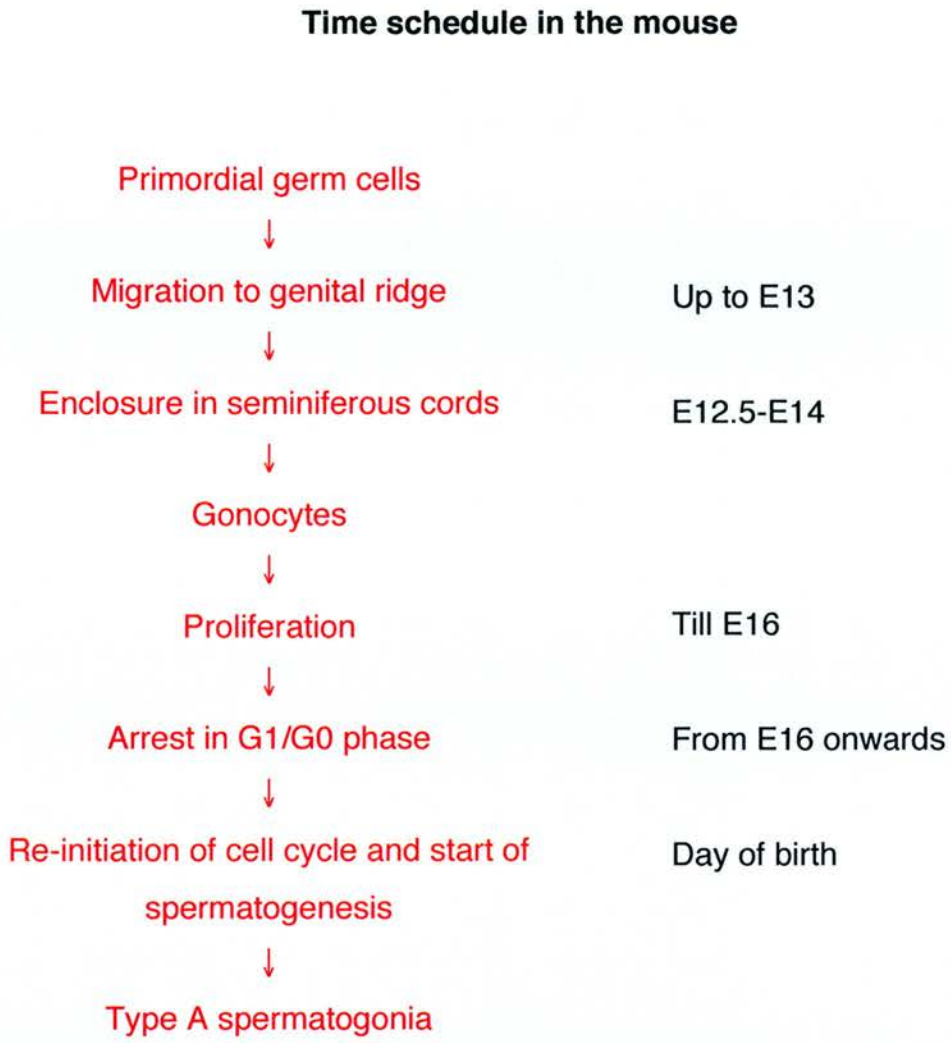


Figure 4.1 Scheme of the development of the spermatogenic lineage from primordial germ cells to type A spermatogonia in the mouse. Adapted from de Rooij (1998).

In the adult male, once type A spermatogonia are formed, further maturation of germ cells occurs in three distinct phases, a proliferative phase where spermatogonia undergo several mitotic divisions culminating in the onset of meiosis and the differentiation into spermatocytes. After a lengthy meiotic prophase, the final meiotic division results in the production of haploid spermatids. The final phase, termed spermiogenesis is where the spermatids undergo structural remodelling. The nuclear material becomes compacted and the spermatid elongates to form the spermatozoa

which will be released from the lumen of the tubule (Cooke and Saunders, 2002; de Rooij, 2001; Sharpe, 1994).

4.1.2. Gene expression in spermatogonia

The testis contains many different types of spermatogonia with a small fraction of these being stem cells. In recent years, the expression of specific markers has been shown in the testis and the localisation pattern of such markers has been useful in the identification of different populations of spermatogonia.

4.1.2.1. Oct-4

Oct-4 (also known as Oct-3) is a transcription factor that belongs to class V of the POU (Pit, Oct, Unc) homeodomain family of transcriptional regulators (Rosner et al., 1990; Scholer et al., 1990). Oct-4 belongs to the sub-group of octamer-binding proteins, which bind to promoter and enhancer regions of various genes via the POU domain. Most POU domain transcription factors are developmentally-regulated in mice (Hansis et al., 2000). Oct-4 is expressed in murine embryonic cells, germ cells (Palmieri et al., 1994) and in cultured embryonic stem cells (Rosner et al., 1990) but is absent from trophectoderm cells (Palmieri et al., 1994). Human OCT-4 orthologs have been cloned and show high homology to murine Oct-4. Oct-4 is one of the factors known to play an important role in the establishment and maintenance of the mammalian totipotent cell population (Hansis et al., 2000; Nichols et al., 1998). As totipotent cells differentiate, Oct-4 is downregulated (Pesce and Scholer, 2000). Oct-4 expression has been detected in the postnatal mouse testis and it is thought to be involved in the development of germ cells. A transgenic mouse created by inserting a construct containing the Oct-4 genomic fragment with a GFP reporter gene was used to demonstrate the germ-line expression of Oct-4. The pattern of Oct-4 protein expression was shown to be expressed in the mitotically arrested prospermatogonia in testes at late gestation and at birth in the mouse (Yoshimizu et al., 1999). This expression has also been reported to continue into type A spermatogonia and then be down-regulated in type B spermatogonia and in spermatocytes in the adult testis (Pesce et al., 1998).

As well as Oct-4, another transcription factor, Stat3 participates in the process of embryonic stem cell renewal. It was thought that Oct-4/Stat3 were the main regulatory factors involved in the process of ES cell self renewal but in 2003, studies described another transcription factor, which was named "Nanog", and suggested that this factor together with Oct-4 and Stat3 plays a crucial role in the process. In particular, it was shown that Nanog overexpression reduces the differentiation potential of mouse embryonic stem cells while Nanog deletion pushes ES cells to differentiate (Chambers et al., 2003; Mitsui et al., 2003). It has been recently reported that suppression of Oct-4 using RNA interference can affect the choice of lineage of mouse embryonic stem cells, pushing the stem cells towards a differentiated phenotype (Velkey and O'Shea, 2003).

4.1.2.2. c-kit

Studies investigating the role of the tyrosine kinase receptor c-kit and its ligand stem cell factor (SCF, also known as Kit ligand) have shown the importance of these factors for the normal development of spermatogonia. The c-kit gene is found on mouse chromosome 5 and human chromosome 4 (Loveland and Schlatt, 1997). During development, c-kit mRNA is expressed in PGCs from 7dpc in the mouse indicating a role for c-kit/SCF in PGC migration (Manova et al., 1993). In mice, mutations in genes encoding either c-kit or SCF result in infertility due to a loss of germ cells (Besmer et al., 1993). In the postnatal testis, c-kit mRNA is found in spermatogonia, spermatocytes and round spermatids as well as in the Leydig cells (Manova et al., 1993). When c-kit binds to its ligand SCF, the receptor dimerises and becomes autophosphorylated, initiating a signalling cascade which induces cell growth and differentiation (Loveland and Schlatt, 1997). Two forms of SCF have been identified, a soluble and a membrane bound form, both expressed by the Sertoli cells (Rossi et al., 1991). In the mouse testis at birth, the membrane bound SCF is the most abundant form found but as development progresses, the soluble form becomes more prevalent (Manova et al., 1993). SCF is encoded at the Sl locus by nine exons in the mouse, rat and human (Brannan et al., 1992; Martin et al., 1990). It has been shown that interactions between SCF/c-kit can modulate primordial germ cell adhesion to somatic cells. It has been suggested that the role played by SCF in the

promotion of survival, proliferation and migration of these cells *in vitro* and *in vivo*, might depend on the ability of the membrane-bound form of this cytokine to mediate primordial germ cell adhesion to the Sertoli cells (Pesce et al., 1997).

The importance of c-kit, which is encoded at the white spotting (W) locus (Chabot et al., 1988; Manova et al., 1990; Matsui et al., 1990), was first discovered when an anti-c-kit antibody (ACK2) was injected into adult male mice. This caused a reduction in the number of differentiated spermatogonia (Packer et al., 1995). Using the vitamin A deficient mouse which only contain A type spermatogonia, (van Pelt et al., 1996), c-kit mRNA and protein expression in differentiated and undifferentiated type A spermatogonia was investigated (Schrans-Stassen et al., 1999). It was shown that A_s and A_{pr} spermatogonia are c-kit negative while the A_{al} and A_1 spermatogonia are c-kit positive. The importance of the c-kit system in the germ cell is discussed further in Chapter One, section 1.4.

4.1.2.3. Dazl

Dazl encodes an RNA binding protein essential for spermatogenesis (Ruggiu et al., 1997; Schrans-Stassen et al., 2001). Dazl is expressed in the mouse testis from embryonic day 12.5 and in the adult testis, expression of Dazl is seen mainly in the spermatogonia and primary spermatocytes (Ruggiu et al., 1997). Adult Dazl knockout mice are infertile and males lack germ cells apart from spermatogonia due to failure of the A_{al} spermatogonia to differentiate into A_1 spermatogonia (Ruggiu et al., 1997). A recent report showed that germ cells from Dazl knockout males were unable to progress beyond the leptotene stage of meiotic prophase I during the first wave of spermatogenesis (Saunders et al., 2003).

4.1.2.4. GFRα-1

Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor-β family (Lin et al., 1993). GDNF is produced by the Sertoli cells and is thought to regulate the fate of undifferentiated spermatogonia, including spermatogonial stem cells (Meng et al., 2000). GDNF binds to and activates a receptor complex consisting of GDNF family receptor (GFR) and Ret receptor

tyrosine kinase (Airaksinen and Saarma, 2002; Airaksinen et al., 1999; Saarma and Sariola, 1999). A GDNF dimer first binds to a dimer of GFR-α1, which dimerises two molecules of Ret inducing transphosphorylation of the tyrosine kinase domains (Sariola and Meng, 2003). In the testis, GDNF is expressed by the Sertoli cells. GFRα-1 mRNA expression is restricted to undifferentiated spermatogonia and like GDNF, its receptors show the highest levels of expression postnatally, remaining detectable in the adult testis (Meng et al., 2000). It has been observed that GFRα-1 positive spermatogonia are rare and are almost never found in close proximity to other GFRα-1 positive spermatogonia (Dettin et al., 2003; von Schonfeldt et al., 2004).

Mice which have targeted deletions of GDNF (Moore et al., 1996), GFR-α1 (Enomoto et al., 1998) or Ret (Schuchardt et al., 1994) show similar phenotypes. They exhibit normal testicular morphology but do not survive for more than a day after birth due to severe defects in the renal system. The GDNF heterozygote mice are fertile, but show degeneration of spermatogenesis resulting in a Sertoli-cell-only histology in the tubules (Meng et al., 2001). Transgenic mice which overexpress GDNF in the testes under the control of the translation elongation factor 1 promoter are infertile (Meng et al., 2001), this promoter targets transgene expression specifically to male germ line cells (Mizushima and Nagata, 1990). Overexpression of GDNF results in an accumulation of undifferentiated spermatogonia from 2 weeks of age (Meng et al., 2000). These undifferentiated cells form large clusters, which express the germ line marker TRA98 and the spermatogonial marker EE2. No expression of Sertoli or Leydig cell markers is seen and the clusters do not express c-kit, which is a marker of differentiated spermatogonia (Meng et al., 2000; Schrans-Stassen et al., 1999). From this information, it has been suggested that when GDNF levels in the testis are low, spermatogonia will enter a differentiation pathway however, at high levels, spermatogonia mainly self renew resulting in increased numbers of stem spermatogonia (Meng et al., 2000).

4.1.2.5. Oestrogen receptor β

Oestrogens have been shown to inhibit the development of gonocytes, Leydig and Sertoli cells in fetal rat testis *in vitro* (Lassurguere et al., 2003) however, a positive direct effect of oestrogens on gonocyte development has been shown in purified gonocyte cultures (Li et al., 1997). It has been previously shown that ERβ is expressed in the germ cells of the rodent testis (Saunders et al., 1998; Zhou et al., 2002). A recent study by Delbes et al. (2004) has suggested that endogenous oestrogens can inhibit germ cell growth in the male as βERKO neonates have more gonocytes in their testis than wild-type littermates. This study also proposed that oestrogens exert their effects on male fetal gametogenesis via ERβ (Delbes et al., 2004).

4.1.3. Identification and enrichment of spermatogonial stem cells

Development of the germ cell transplantation technique by Brinster and colleagues in 1994 (described in detail in section 1.8) has provided a functional test for the identification of spermatogonial stem cells (Brinster and Zimmermann, 1994; Brinster and Avarbock, 1994). The efficiency of the technique has been significantly improved by using populations of germ cells enriched for stem cells (Shinohara et al., 2000). Examples include populations of germ cells obtained from young animals or those isolated using immunobeads (Giuili et al., 2002; Shinohara et al., 1999; Shinohara and Brinster, 2000). For example, in 1999 Shinohara et al. showed that isolating germ cells expressing α6/β1 integrins, which were thought to be putative stem cells, enhanced the efficiency of transplantation 166 fold. The same study showed that selecting cells that expressed c-kit did not result in stem cell enrichment, providing evidence that c-kit is a marker of differentiated spermatogonia but not spermatogonial stem cells (Shinohara et al., 1999).

4.1.4. Germ cell lines

Several cell lines have been established from germ cells at various stages of differentiation. The GC-1 and GC-2 cell lines were prepared by immortalisation of spermatogonia and spermatocytes respectively (Hofmann et al., 1994; Hofmann et al., 1992) with the GC-2 line being reported to undergo meiosis *in vitro* (Hofmann et

al., 1994). Although subsequent investigation of the cell line showed that the cells were no longer able to differentiate *in vitro* (Wolkowicz et al., 1996). A further germ cell line, GC-4 has been characterised as being at the stage between preleptotene and early pachytene spermatocytes (Tascou et al., 2000). To date, only two reports of cell lines being established from rodent spermatogonial stem cells exist. In the first, a cell line was established by transfecting A_s (stem cells), A_{pr} and A_{al} spermatogonia from the rat with the SV40 large T antigen. Two cell lines were established and upon characterisation, were found to express the germ cell markers Hsp90 and Oct-4. No expression of c-kit, normally expressed in differentiated spermatogonia could be detected in either cell line (van Pelt et al., 2002). These cells were transplanted into infertile mice and were seen to migrate to the basement membrane and re-colonise the seminiferous epithelium. The second report came in 2002, with Feng et al. showing that retroviral infection of mouse spermatogonia with telomerase could immortalise the cells. The cells were shown to express the germ cell markers Oct-4 and culture with SCF induced the cells to differentiate. However, no transplantation studies have been carried out in these cells, which would be a test of whether they have maintained their stem cell characteristics (Feng et al., 2002).

4.1.5. RNA interference

RNA interference is a powerful tool that can cause sequence specific gene silencing (Elbashir et al., 2001). The development of vector-based delivery systems has allowed more stable expression of siRNAs. When placed in a vector, siRNAs are expressed as fold-back hairpin–loop structures that give rise to siRNAs after intracellular processing (Tuschl, 2002). Transfection of plasmid DNA, rather than synthetic siRNAs, is advantageous in many ways, overcoming problems with RNase contamination and the costs of chemically synthesized siRNAs or siRNA transcription kits. By using a vector based delivery system, longer-term knockdown can also be achieved.

Lipid-based transfection reagents are typically used for siRNA delivery in immortalized cell lines. However these reagents have been shown to be inefficient for siRNA delivery to most primary cell types and to cells grown in suspension.

Electroporation has been used as an alternative to chemical transfection to deliver siRNAs into primary cells in suspension and other hard-to-transfect cell types (Walters and Jelinek, 2002) and involves applying an electric field pulse to induce the formation of microscopic pores in the cell membrane which allow molecules, ions, and water to traverse the membrane (Hamm et al., 2002). Under specific pulse conditions, the pores reseal and the "electroporated" cells recover and resume growth. A distinct advantage of electroporation over chemical methods is that it is not dependent on cell division (Chu et al., 1987; Hamm et al., 2002).

Most existing electroporation protocols were developed to deliver plasmid DNA to the cell nucleus but these protocols often result in high cell mortality (Chu et al., 1987; Hamm et al., 2002). Since siRNAs only need to be delivered to the cytoplasm, milder electroporation conditions can be used that minimize cell death but still ensuring highly efficient siRNA delivery (Gehl, 2003). A report by Walters and Jelinek (2002) investigated the knockdown efficiency of various transfection methods on adherent and non-adherent cells, the study concluded that electroporation was the most effective method of transfection for cells in suspension (Walters and Jelinek, 2002).

4.1.6. Aims

The studies described in this chapter set out to initially investigate the expression profile of 2 germ cell lines, GC-1 and GC-2. Following this, establishment of a reliable method for the isolation of highly purified murine spermatogonial stem cells was attempted. Cells were characterised by investigating whether they expressed stem cell markers. The level of expression of ERβ mRNA in total testis extracts compared with isolated stem cells was determined using Q RT-PCR. RNA interference was used to investigate the impact of targeted gene knockdown on the differentiation status of the germ cells.

4.2. Materials and Methods

4.2.1. Cell culture

The GC-1 and GC-2 germ cell lines were obtained from ECACC. The cells were maintained in culture at 37°C in 5% (v/v) CO_2 in complete media (sections 2.3.1 and 2.14.2). Passaging of cells was performed every 3-4 days by trypsinisation (section 2.3.2).

4.2.2. Sterile isolation of spermatogonial stem cells

Sterile instruments and solutions were used throughout the procedure.

The animals were sacrificed by cervical dislocation or by inhalation of CO_2. The testes were removed from the animal and placed into Hanks Buffered Salt Solution (HBSS; Gibco). Under a dissection microscope, the tunica albuginae was removed, the tubules carefully pulled apart and placed in 4.5mls HBSS. All subsequent steps were performed in a class II tissue culture hood and all incubations were carried out at 34°C on a roller.

To the tubules, 500µl collagenase type IV (0.01g in 1ml HBSS) (Sigma) was added and incubated for 10 minutes. After incubation, the tubules were shaken gently before incubating for a further 5 minutes at 34°C; 50µl DNase 1 (0.007g in 1ml HBSS; Roche) was added to the tubules and incubated for 5 minutes. The cell suspension was centrifuged for 5 minutes at 500g, the supernatant removed and discarded and the cell pellet re-suspended in 4mls HBSS. This step was repeated twice, 0.5ml trypsin (Gibco) and 0.5ml EDTA (Gibco) was added and the cell suspension incubated for 10 minutes. The cells were shaken to obtain a single cell suspension and 0.5ml fetal calf serum added to stop any further action of trypsin. The cells were filtered through a 70µm mesh filter (Falcon), centrifuged at 500g for 5 minutes and resuspended in 10mls of supplemented EBSS (section 2.17.2) and a cell count performed.

4.2.3. Immunomagnetic bead sorting

Isolation of spermatogonial stem cells was performed using specific antibodies and magnetic beads coated with secondary antibodies based on the method described by van der Wee in 2001(van der Wee et al., 2001). As discussed earlier (section 4.1.3), spermatogonial stem cells are known to express the cell surface markers α-6 integrin and GFRα-1. By using antibodies directed against these proteins, populations of cells enhanced or depleted for these markers were collected. Cells, pre-incubated with antibodies were removed from the cell suspension using a secondary antibody which was conjugated to magnetic beads. By incubating the cells with the immunomagnetic beads and placing in a magnet, beads with the attached cells adhere to the magnet allowing removal from the total cell population. In this study M-450 sheep anti-mouse IgG and M-450 Sheep anti-rat IgG Dynabeads® (Dynal) were used.

4.2.3.1. Cell separation – indirect technique

Cells were isolated from single cell suspensions as this increased the efficiency and specificity of the sorting. The cells were adjusted to 10 million cells/ml in supplemented EBSS (section 2.13.3). A sample of unsorted cells was taken at this stage and stored for further analysis. The cell suspension was incubated with the specific primary antibody; Table 4.1 shows antibodies used.

Antibody	Species	Supplier	Dynabeads
α-6 integrin	Rat	BD Biosciences	Sheep anti-rat IgG
GFR-α-1	Rabbit	Autogen Bioclear	Sheep anti-rabbit IgG

Table 4.1 Antibodies and dynabeads used for immunomagnetic cell sorting

Figure 4.2 shows the steps involved in the sorting process using α-6 integrin as an example. The primary antibody was added in excess to block all antigen-binding sites on the cell surface. The cells were incubated in 2μg of primary antibody for 30 minutes at 4°C on a rocking platform. After incubation, the cells were centrifuged at 1000 rpm for 10 minutes and the supernatant containing unbound immunoglobulin,

was discarded. Cells were resuspended in PBS containing 0.1% BSA; this step was repeated twice to thoroughly wash the cells of any excess antibody. The cells were incubated with the appropriate dynabeads (Table 4.1); $1x10^7$ dynabeads per ml of sample were used giving approximately 4 beads per target cell. Before adding to the cell suspension the required volume of dynabeads were washed as follows: beads were vortexed and transferred into a washing tube and placed in the magnet (Dynal) for 2 minutes. The buffer was removed and the tube was removed from the magnet. An excess volume of PBS containing 0.1% BSA (Sigma) was added and the beads resuspended. This washing step was repeated twice and the washed beads were resuspended in PBS containing 0.1% BSA (Sigma) to a concentration of 1×10^7 beads per ml. The washed beads were added to the antibody-covered cells and the mixture was incubated for 30 minutes at 4°C on a rocking platform.

After the incubation period, the cells/dynabead suspension was placed in the magnet and allowed to stand for 2 minutes. The supernatant, which contained cells that remained unbound to the beads (unbound fraction), was removed and stored. The cells which were antibody/bead positive (bound fraction) were resuspended in PBS containing 0.1% BSA and stored for further analysis.

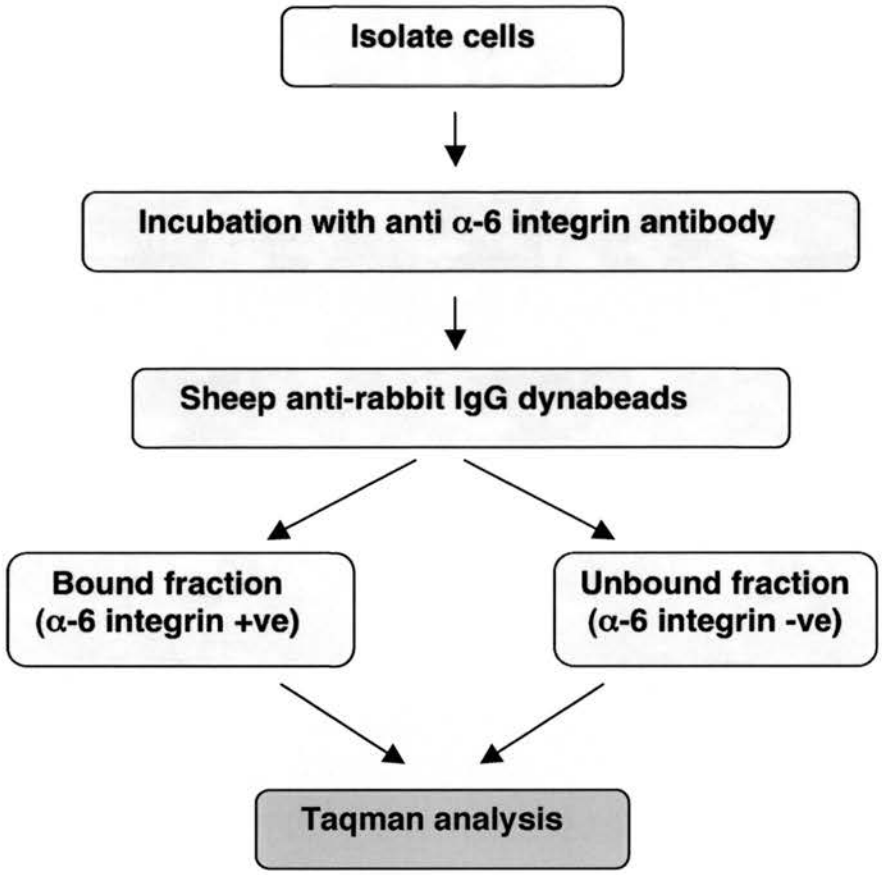


Figure 4.2 Immunomagnetic sorting of spermatogonial stem cells using anti α-6 integrin.

4.2.4. RNA extraction from cultured cells, d8 mouse testis and immunomagnetically sorted testicular cell populations

GC-1 and GC-2 cells were trypsinised as in section 2.3.2 and the resulting cell pellet resuspended in TRI reagent as described in section 2.5. RNA was extracted from the cells (section 2.5) for RT-PCR (section 4.2.5).

To investigate the expression of germ cell mRNA's expressed throughout the first wave of spermatogenesis, RNA was extracted from testes obtained from wild type mice aged from d0 to adult as described in section 2.5 and used for RT-PCR using germ cell specific primers.

Sorted cells obtained through immunomagnetic bead sorting were centrifuged for 10 minutes at 1000 rpm. The supernatant was removed and 100μl of TRI reagent (Sigma) was added. The samples were vortexed and homogenised before RNA was isolated using the procedure detailed in section 2.5.

4.2.5. RT-PCR and nested RT-PCR

Oligo dT primed cDNA was prepared using standard procedures as in section 2.6. RT-PCR was carried out as detailed in section 2.6.2.2 with 40 cycles of amplification using RedTaq DNA polymerase (Sigma). Primer sequences used for RT-PCR are shown in Table 4.2 PCR products were run on a 2% agarose gel (section 2.7).

Gene	5' Primer	3' Primer	Product size (bp)
c-kit	ATGCGTGTGTC TATGCGTGTGG	GGATTGGCAGC ATTACATAAGG	275
Oct-4	GAAGCCGACA ACAATGAGAACC	GCTCCAGGTTC TCTTGTCTACC	517
Dazl	ATCCTCCTTAT CCAAGTTCACC	ACTGTCTGTAT GCTTCGGTCC	261
RBM	GTAATTGCCAT AGTCACAGTATCC	AAGCAGAGCAA GAAGTGGTCC	347
ERβ	CCAATGTGCTA GTGAGCCG	AACTCACGGAA CCGTGCCG	393
LDH-C4	CCCTTGTTGACGCT GATACGA	TTAAGGACCTCATAG CCGCCT	540
GFR α-1	TCCTATGAAGAACG AGAGAGGC	AAGCAGTCTTCCAGG TCATTGC	285
Nanog	AGGGTCTGCTACTG AGATGCTCTG	CAACCACTGGTTTTT CTGCCACCG	363

Table 4.2 RT-PCR primer sequences

Because of the low abundance of target material, nested PCR was carried out on the sorted cell populations. Nested PCR involves re-amplification of a PCR product using primers designed to fall within the original amplified region. Table 4.3 shows sequences of the nested primers used in this study.

The nested RT-PCR reaction was set up as follows:

10x PCR buffer	2.5µl
dNTP's	0.5µl
Nested primer (Sense)	0.5µl
Nested primer (Antisense)	0.5µl
RedTaq DNA Polymerase	0.5µl
H_20	19µl
Initial PCR product	1µl
TOTAL VOLUME	25µl

Samples were run on the PCR thermocycler according to the following cycling conditions:

94°C	2 minutes	
94°C 55°C 72°C	1 minute 30 seconds 30 seconds	15 cycles
72°C	10 minutes	

Products were run on a 2% agarose gel (section) alongside 100bp DNA ladder (Promega)

Gene	5' primer	3' primer	Product size (bp)
c-kit	CCAAGACGTA ACACGTTCTG	GCAGCATTACA TAAGGTCTA	194
Oct-4	GAGATATGCAA ATCGGAGACC	CAGAGGAAAGG ATACAGCC	327
Dazl	CAAGTTCACCA GTTCAGGTC	CATTTCCAGAA GCTGGAGC	208
RBM	GAAGTGGTCCT TCATGTGAAG	GGCCATAATCC CTTGGTAAG	275
ERβ	TGCTAGTGAGC CGTCC	CCAAAGATTTCC AGAATCC	306
GFRα-1	CCACTGCCTGA GTCTGC	AGGTCATTGCCGC TGTTGC	243

Table 4.3 Nested RT-PCR primer sequences

4.2.6. TaqMan Q-RT-PCR

Quantitative RT- PCR (section 2.12) was carried out on the immunomagnetic bead sorted cells using primers and probes designed for ERβ (section 2.12.2) and Assay-on Demand primer/probe systems for both Oct-4 and c-kit (Applied Biosystems, section 2.13.4). Synthesis of cDNA, the Q-RT-PCR reaction and subsequent analysis was carried out as detailed in sections 2.12.5, 2.12.6 and 2.12.7 respectively.

4.2.7. Immunohistochemistry

Expression of several germ cell markers was investigated using sections taken from Bouins-fixed day 8 mouse testis (section 2.1.1). Immunohistochemistry was carried out as in section 2.2.

4.2.8. Culture of sorted cell populations

Following isolation, the spermatogonial stem cells were counted and 4 x 10^6 cells were seeded per 25cm^2 flask. The cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal calf serum, 100mM non-essential amino acids, 6mM glutamine, 50μg/ml penicillin/streptomycin and 0.5mM sodium pyruvate. The cells were maintained at 34°C with 5% CO_2 in a humidified incubator.

To investigate the impact of feeder cells on the maintenance of spermatogonial stem cells, the cells were co-cultured on a layer of STO cells (Ware and Axelrad, 1972), which were obtained from ECACC. These cells were treated with mitomycin C to inhibit cell division (section 4.2.8.1). Spermatogonial stem cells were plated at a density of 1 x 10^5 cells/ml on top of the feeder layers.

Evaluation of the effect of soluble factors on the maintenance and differentiation status of spermatogonial stem cells was carried out by the addition of the growth factors leukaemia inhibitory factor (LIF) and stem cell factor (SCF) and the steroid hormone 17β oestradiol to the cultured cells. LIF (murine recombinant, Chemicon) and SCF (Chemicon) were added to the culture medium at a concentration of 50ng/ml and 30ng/ml respectively, 17β oestradiol was added to the cultures at a final concentration of 100nM. All three were added to the cultures at the start of the

culture period, termed day 0. The medium was replaced with fresh medium containing the supplements every 2 days and the cultures harvested after 7 days for RNA extraction and TaqMan analysis.

4.2.8.1. Mitomycin C treatment

Mitomycin C is a potent inhibitor of DNA synthesis and nuclear division and was used to block cell division during the production of feeder layers for the culture of spermatogonial stem cells. STO cells (section 2.3.1) were used as feeder cells and were 70-80% confluent at the time of treatment. Mitomycin C (Sigma) was dissolved in water to a final concentration of 1mg/ml and protected from light. Cell monolayers were washed in PBS and 10ml fresh complete medium containing a final concentration of 200μg/ml mitomycin C was added per T75 flask. The cells were incubated at 37°C for 2.5 hours. After treatment, the media was removed and the cells washed with PBS before trypsinisation (section 2.3.2) and counting. The cells were plated onto gelatin coated flasks or dishes which were prepared by adding enough of 0.1% gelatin to cover the base of the vessel. The gelatin was removed after 1 hours of incubation at room temperature. Cells from one T75 flask were plated onto two gelatinised T25 flasks or four 6cm gelatinised dishes and allowed to attach overnight forming a feeder monolayer. Alternatively, the cells were frozen down after mitomycin C treatment and trypsinisation in freezing medium (section 2.13.2) and stored in liquid nitrogen for future use.

4.2.9. RNA interference

4.2.9.1. Preparation of plasmid vectors containing short hairpin RNA

The pSilencer 3.0 H1 vector (Ambion, section 2.11.5) was used to express siRNA's specific for murine Oct-4 and ERβ. The Oct-4 hairpin oligonucleotides were designed based on the 19mer hairpin sequence published by Velkey and O'Shea (2003) and are shown in Table 4.4. The ERβ sequence (Table 4.4) was based on a linear siRNA sequence that had been shown to give knockdown in the SK11 cell line. Details of the construction of the vector constructs are given in Chapter 2, section 2.11.5.

Gene	Sense oligonucleotide 5'→ 3'	Antisense oligonucleotide 5'→ 3'
Oct-4	GATCCGTTTCTGAAGTGCCCG AAGTTGAAGAGACTTCGGGCA CTTCAGAAACTTTTTTGGAAA	AGCTTTTCCAAAAAAGTTTCTGAA GTGCCCGAAGTCTCTTGAACTTC GGGCACTTCAGAAACG
ERβ	GATCCAAGAAGATAATGGTCA AGCTTCTCAAGAGAAAGCTTG CCATTATCTTCTTTTTTGGAAA	AGCTTTTCCAAAAAAGAAGATAA TGGTCAAGCTTTCTCTTGAGAAG CTTGACCATTATCTTCG

Table 4.4 Oct-4 and ERβ shRNA oligonucleotide sequences. The 19mer Oct-4 sequence specific siRNA sequence (sense and antisense) is underlined and shown in red with the loop sequence shown in blue.

The oligonucleotides were annealed and ligated into the pSilencer vector as described in sections 2.11.5.1 and 2.11.5.2. The vector was then transformed into XL-1 Blue competent cells as in section 2.11.5.3. Colonies were picked, grown up overnight in medium and plasmid minipreps prepared as in section 2.8.6. Sequencing was carried out to confirm the correct insertion of the siRNA insert into the plasmid (section 2.9) before CsCl banding of large-scale plasmid preparations (section 2.10) was performed.

4.2.9.2. Transfection of spermatogonial stem cells with an shOct-4 vector using oligofectamine

Transfection of the spermatogonial stem cells with the shOct-4 vector was performed using oligofectamine as described in section 2.11.3.1. RNA was harvested from the cells (section 2.5) 2 or 6 days after transfection and cDNA prepared for TaqMan Q-RT-PCR as described in section 2.12.6.

4.2.9.3. Culture of spermatogonial stem cells with an shOct-4 vector

Sorted cells were cultured in the presence or absence of 20nM shOct-4 for a total of 2 or 6 days. RNA was extracted from the cells and TaqMan quantitative RT-PCR performed on the treated and untreated cells.

4.2.9.4. Electroporation of spermatogonial stem cells with shRNA vectors

Spermatogonial stem cells were centrifuged at 500 rpm for 5 minutes and washed in supplemented EBSS (section 2.15.1) before resuspension in 400µl EBSS (no supplements). 10µg of shRNA vector was diluted in 100µl EBSS and added to the cells. The cell/shRNA mixture was transferred to a 0.4 cm electroporation cuvette (Bio-Rad) and incubated for 10 minutes at room temperature before electroporation at 250V, 950µF using the Gene PulserII electroporation system (Bio-Rad). The cells were incubated at room temperature for 20 minutes before being transferred into a 6 well plate containing pre-warmed (34°C) DMEM supplemented with 20% FCS, 100mM non essential amino acids, 6mM glutamine, 50µg/ml penicillin/streptomycin and 0.5mM sodium pyruvate. The cells were maintained at 34°C with 5% CO_2 in a humidified incubator. The cells were harvested for RNA extraction 2 or 6 days later as shown in Figure 4.3.

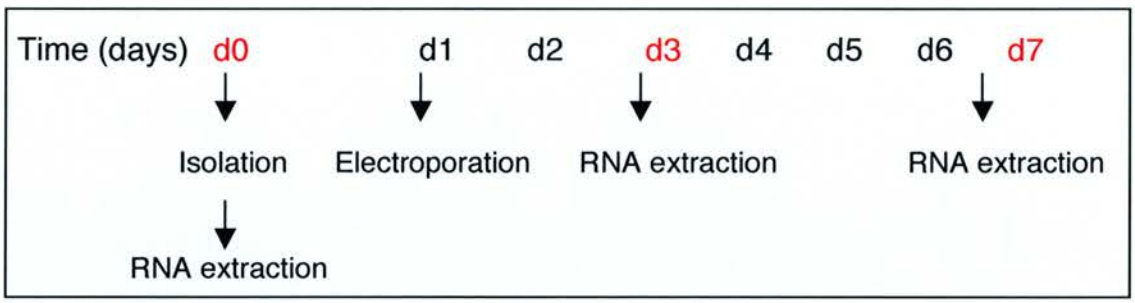


Figure 4.3 Time scale of isolation, electroporation and RNA extraction in spermatogonial stem cells electroporated with an shRNA vector.

4.3. Results

4.3.1. Germ cell development during the first wave of spermatogenesis

The expression of various germ cell markers was investigated in extracts of mouse testis obtained from day of birth until adulthood using RT-PCR with specific primer pairs. Oct-4, c-kit and ERβ mRNAs were all detected at all ages from d0 up to adulthood (Figure 4.4 A, B and C). RBM and Dazl were absent from the d0 and d4 samples with expression of both markers being seen from d8 onwards (Figure 4.4 D and E).

4.3.1.1. Immunohistochemical localisation of germ cell markers in the immature mouse testis.

Protein expression of Oct-4, c-kit, RBM and ERβ was demonstrated in testis sections taken from 8-day-old mice. Immunopositive staining for c-kit (Figure 4.5 B) and RBM (Figure 4.5 C) was germ cell specific in the 8-day-old testis. Immunohistochemistry using an anti ERβ antibody detected staining in the germ cells as well as other cell types, including the Sertoli cells (Figure 4.5 D).

4.3.2. Expression of germ cell markers in GC-1 and GC-2 cells

The expression pattern of a selection of germ cell markers in the GC-1 and GC-2 cell lines was determined by RT-PCR using specific primer pairs in order to compare the cell lines with the in-vivo situation. In each case, adult testis was used as a positive control. Expression of GAPDH a housekeeping gene was used to establish the quality of RNA synthesised was observed in both cell lines (Figure 4.6 H). No expression of Oct-4 was observed in either cell line (Figure 4.6 B). Expression of c-kit, Dazl, RBM and LDH-C4, all germ cell markers was also absent (Figure 4.6 A, C, D and G). The expression of the steroid hormone receptors ERα and ERβ was then investigated. Figure 4.6 E and F show the lack of any expression of the receptors in the GC-1 and GC-2 cell lines.

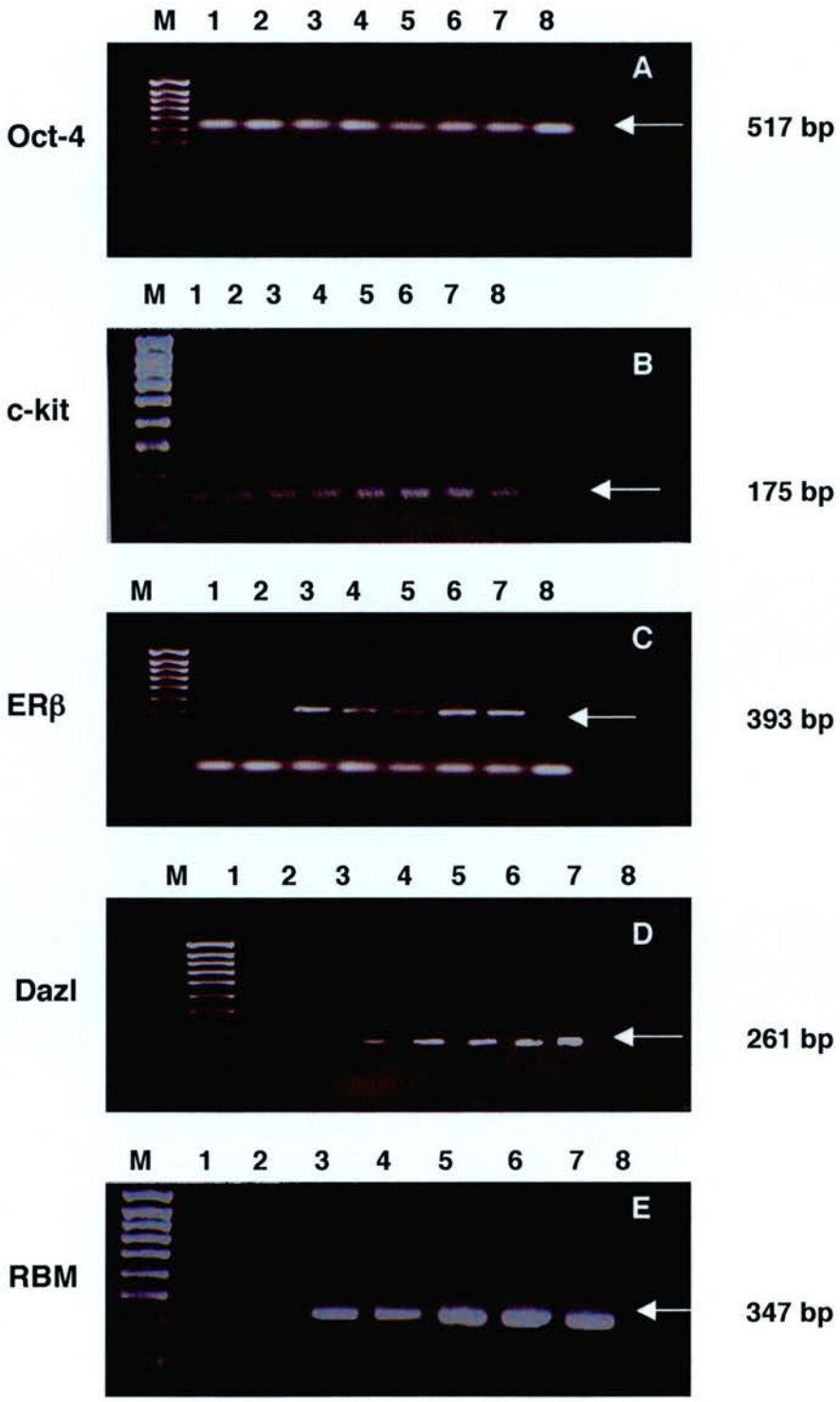


Figure 4.4 RT-PCR of mouse testis from d0-adult using specific primers for (A) Oct-4, (B) c-kit, (C) ERβ, (D) Dazl and (E) RBM. In all cases, M represents DNA ladder, lane 1 represents d0 mouse testis, lane 2 d4, lane 3 d8, lane 4 d12, lane 5 d16, lane 6 d21, lane 7 adult and lane 8 negative control.

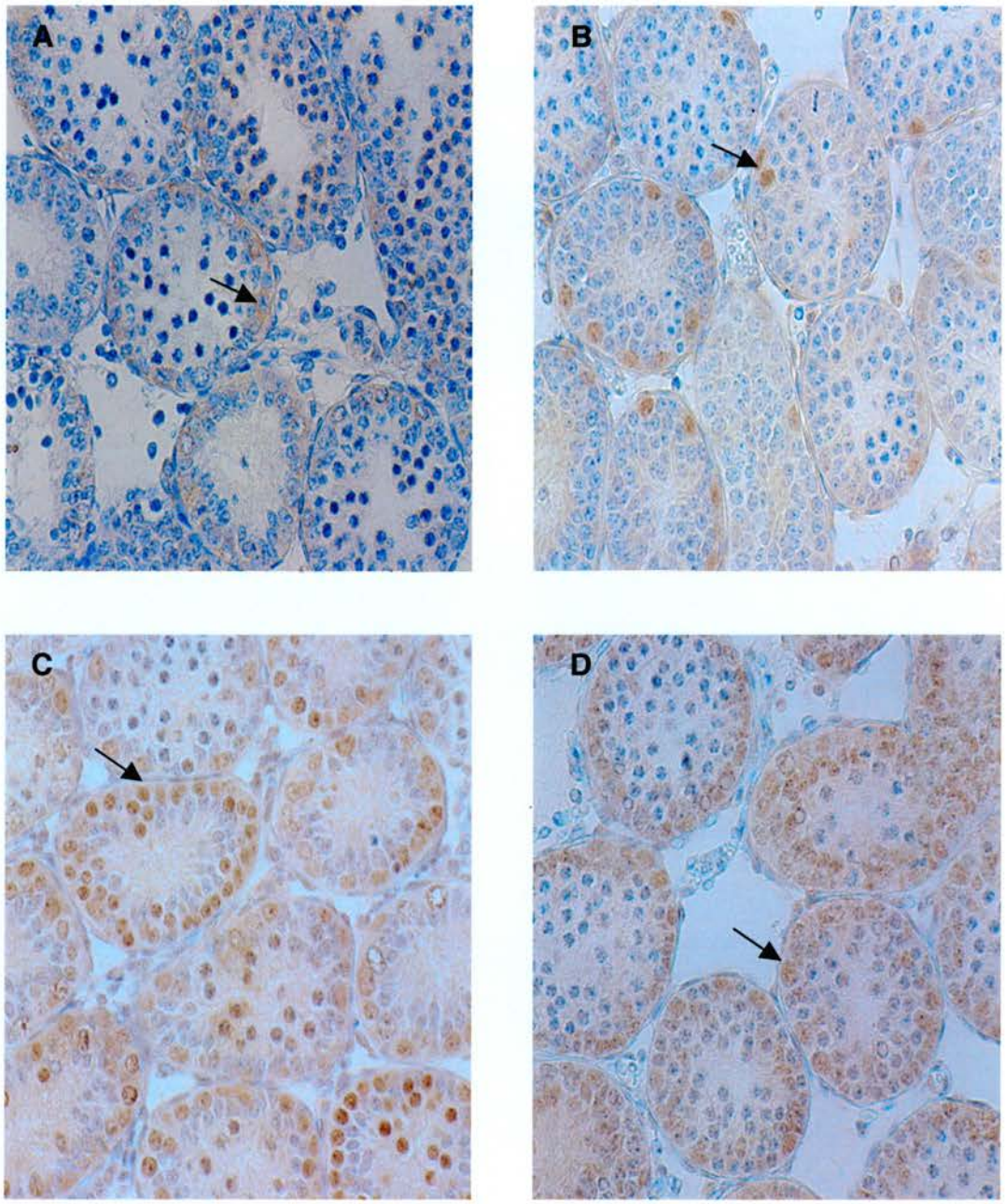


Figure 4.5 Immunohistochemistry on 8-day-old mouse testis using antibodies specific for c-kit (A) Oct-4 (B), RBM (C) and ERβ (D) antibodies. Arrows point to immunopositive germ cells.

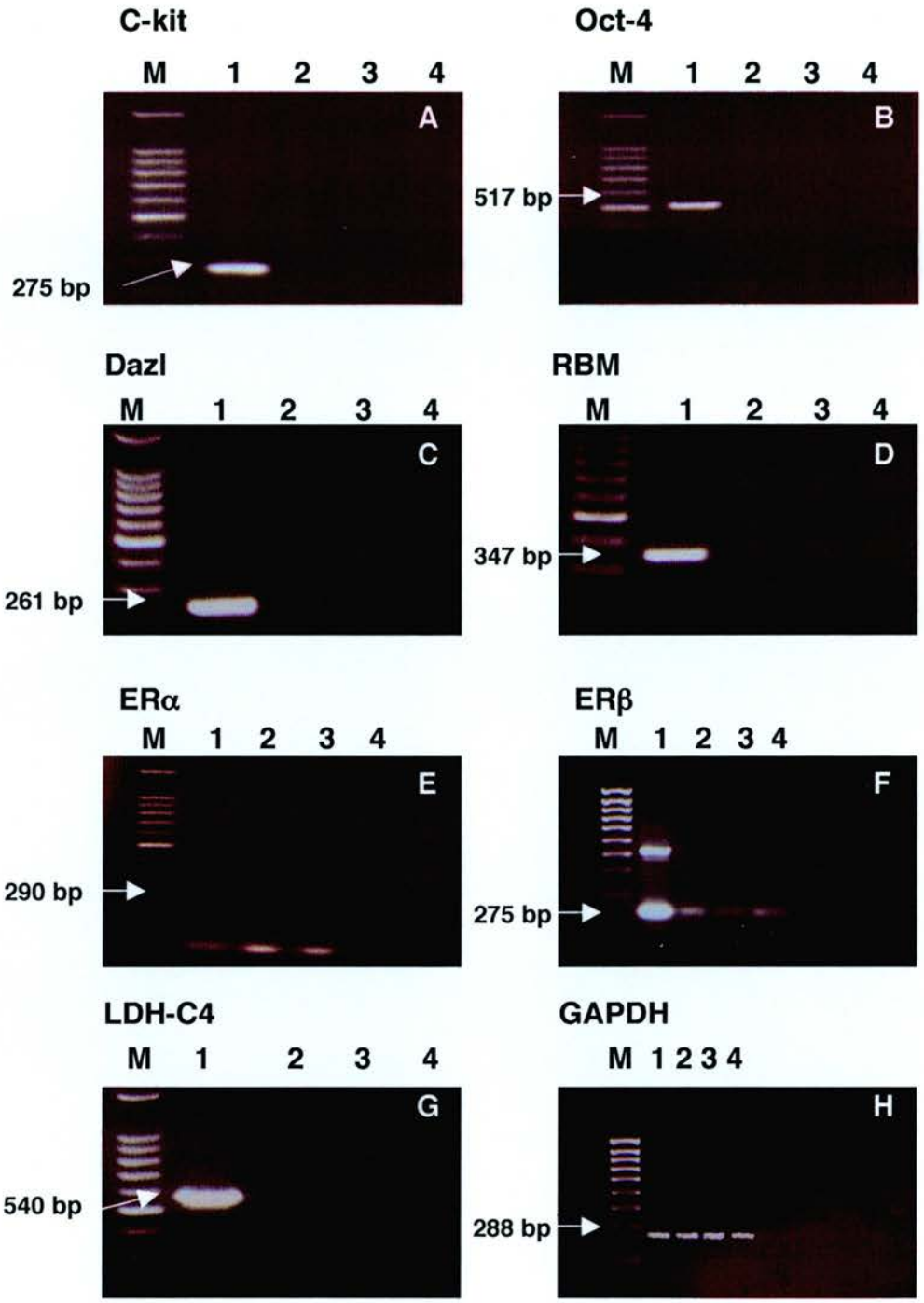


Figure 4.6 Expression of germ cell markers expressed in GC-1 and GC-2 cells by RT-PCR using specific primer pairs. (A) c-kit, (B) Oct-4, (C) Dazl, (D) RBM, (E) ERα, (F) ERβ (G) LDH-C4 and (H) GAPDH. In all cases, M represents DNA ladder, lane 1 is d10 mouse testis, lane 2 is GC-1 cells, lane 3 is GC-2 cells and lane 4 is a negative control sample.

4.3.3. Isolation of spermatogonia using immunomagnetic beads

Once the expression pattern of spermatogonia had been established in the mouse, the aim was to isolate a population of cells enriched for spermatogonial stem cells, allowing more detailed evaluation of the spermatogonial stem cell without any contamination with other cell types. To perform this, a total germ cell population obtained from 8 day old mice was sorted using immunomagnetic bead sorting using either an anti-GFRα-1 antibody or anti α-6 integrin antibody (section 4.2.2). After sorting, a sample of each sorted population was placed on a glass slide and photographed (Figure 4.7)

Due to the low amount of target mRNAs in the sorted cell populations, nested RT-PCR was performed using primer pairs specific for Oct-4, c-kit, GFRα-1 and ERβ. Expression of all the mRNAs was detected in the unsorted control sample (Figure 4.8). In the population of cells, which were bound to the beads, GFRα-1, Oct-4 and ERβ were all expressed. c-kit expression was seen in low levels in the bound population of cells. No expression of Oct-4 or GFRα-1 mRNA expression was detected in the population of germ cells which remained unbound after immunomagnetic sorting. ERβ was observed in the unsorted, bound and unbound cell populations.

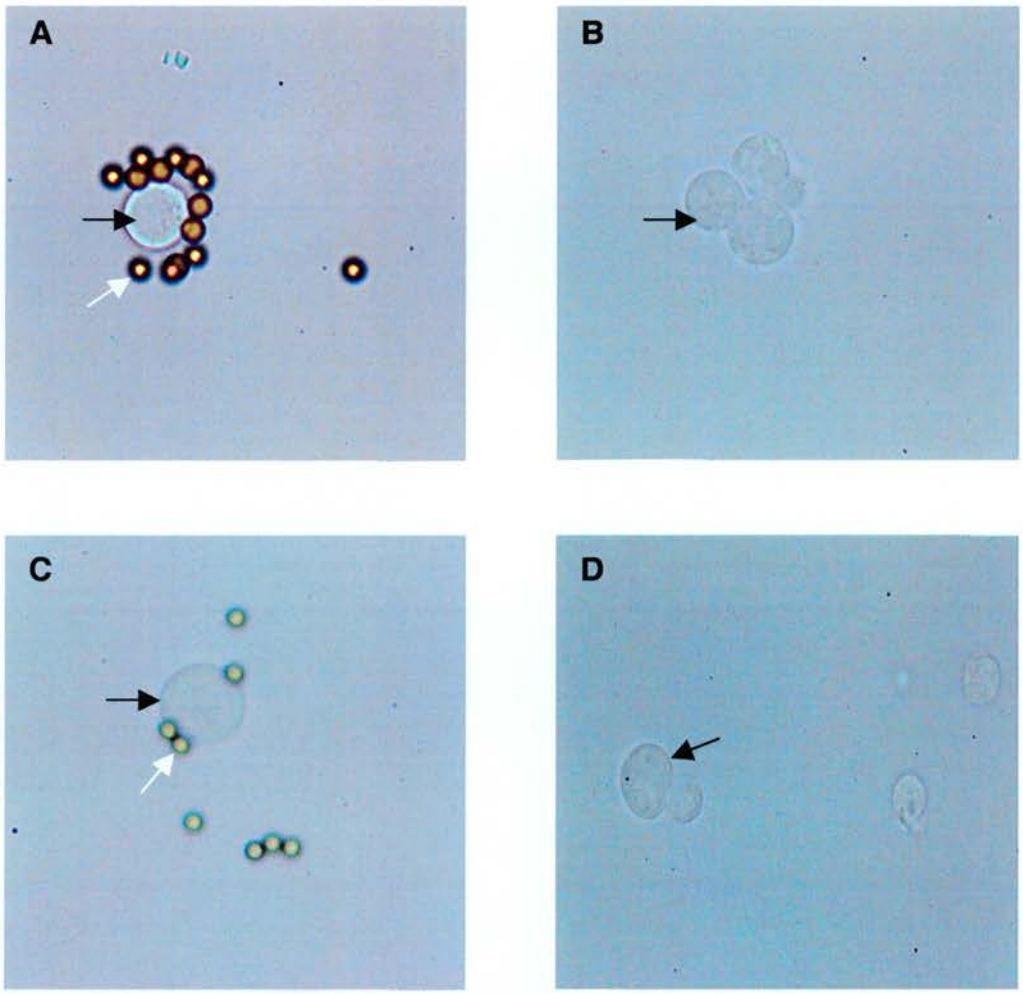


Figure 4.7 A and C show spermatogonial stem cells bound to immunomagnetic beads after incubation with either anti-α6 integrin (A) or anti GFRα1 (C) antibodies. B and D show cells that remained unbound to the immunomagnetic beads after sorting with either anti-α6 integrin (B) or anti GFRα1 (D). Black arrows point to the spermatogonia, white arrows point to immunomagnetic beads.

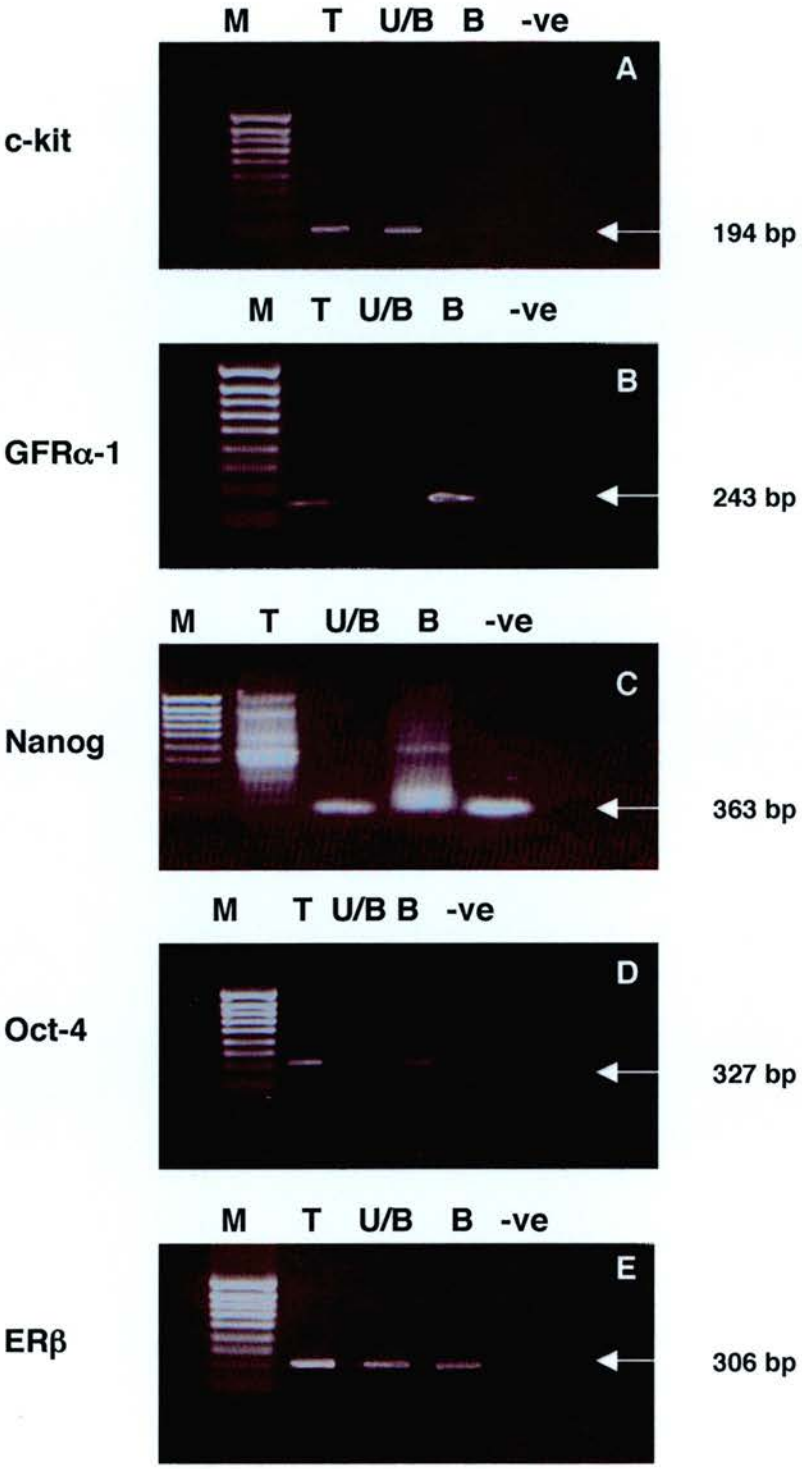


Figure 4.8 RT-PCR analysis of bound, unbound and unsorted spermatogonial cells using primers for (A) c-kit, (B) GFRα-1, (C) Nanog, (D) Oct-4 and (E) ERβ. In all cases, M represents DNA Hyperladder, T shows the unsorted population, U/B an unbound cell population, B the magnetically bound population and –ve is the negative control.

4.3.4. Quantification of mRNAs in sorted compared to unsorted germ cell populations

The results from the semi-quantitative RT-PCR were not sensitive enough to determine differences in mRNA levels between the sorted and unsorted cells. In order to quantify the amount of specific mRNAs expressed in the sorted and unsorted germ cell populations, TaqMan quantitative RT-PCR was performed using RNA obtained from unsorted and immunomagnetic sorted, (bound, α-6 integrin positive and unbound, α-6 negative) cells. RNA extraction and cDNA synthesis was carried out and the expression of Oct-4 and c-kit mRNA was investigated in order to assess the differentiation status of the bound and unbound populations.

4.3.4.1. Oct-4 mRNA expression

Compared to the unsorted population, the bound population showed a greater proportion (1.7 fold increase compared to an unsorted population) of Oct-4 positive cells were present in the sorted, bound, cell population than in the unbound, where the levels of Oct-4 were almost undeterminable (Figure 4.9 panel A).

4.3.4.2. c-kit mRNA expression

Compared to the unsorted population, c-kit expression was greater in the unbound cells (8-fold increase) than in the bound cells where the levels of c-kit were very low, (Figure 4.9 panel B).

4.3.4.3. ERβ mRNA expression

The expression of ERβ mRNA was determined in the same bound, unbound and unsorted cell populations used for the analysis of Oct-4 and c-kit mRNA expression. Figure 4.9 C shows that the bound population of cells (thought to contain the stem cell population) express more ERβ mRNA than the unbound population (2 fold compared to 1.3 fold over control).

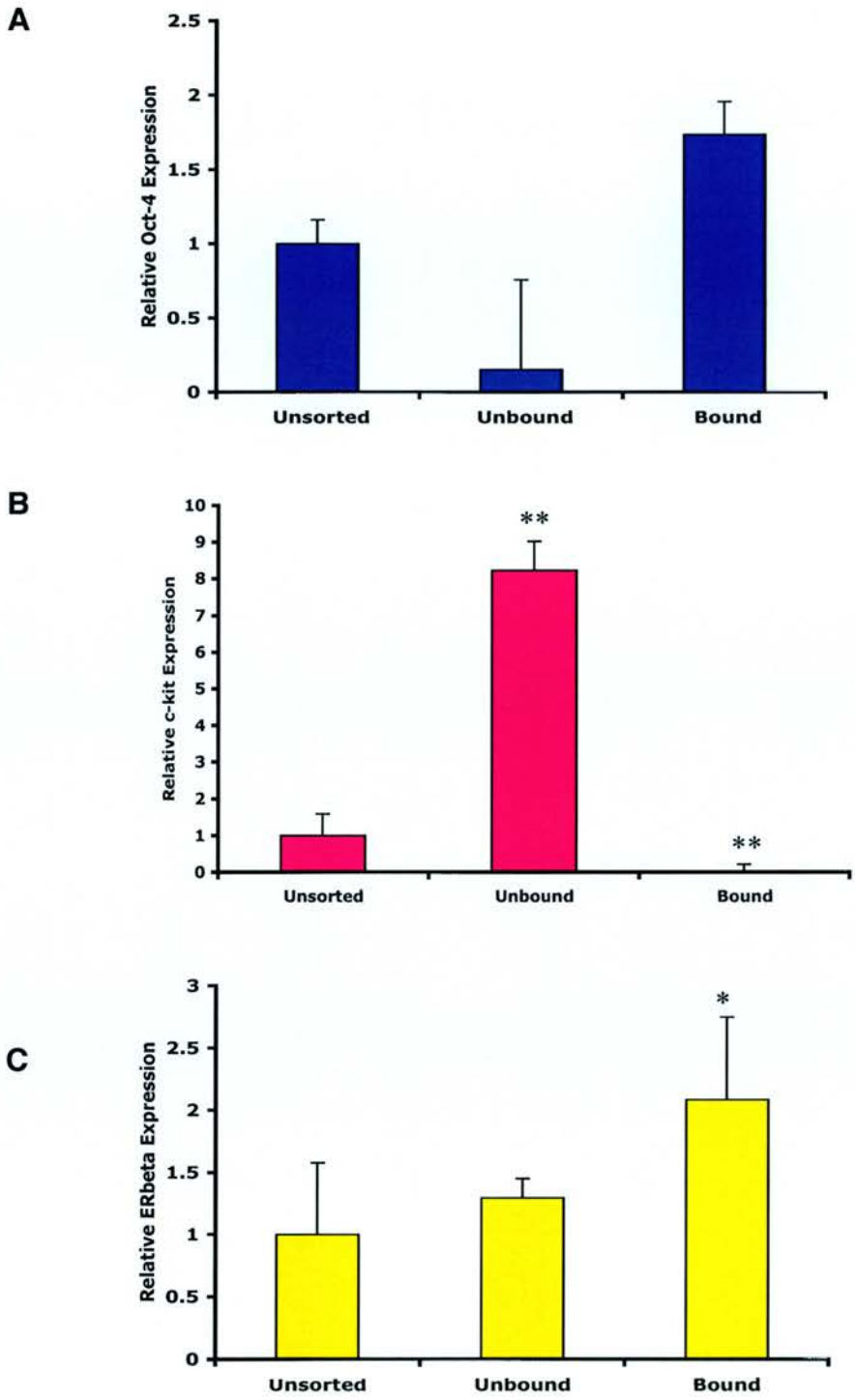


Figure 4.9 Panel A shows the relative amounts of Oct-4 mRNA in bound and unbound spermatogonial stem cells isolated using immunomagnetic beads compared to an unsorted population. The relative amounts of c-kit (panel B) and ERβ (panel C) in the same populations of cells are shown. Results are ± SEM n=3. Statistical analysis was performed by the use of one-way ANOVA. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.001$.

4.3.5. Oct-4, c-kit and ERβ expression in sorted cells after culture

In order to assess treatment effects in the sorted cells, the bound and unbound populations were maintained in culture for up to 7 days (section 4.2.8). Oct-4 (Figure 4. 10A), c-kit (Figure 4.10 B), and ERβ mRNA (Figure 4.10 C) levels as determined by Taqman quantitative RT-PCR were assessed before and after the culture period to check for any culture-induced changes in gene expression. After 7 days of culture, no change in the levels of Oct-4 (Figure 4.10 A), c-kit (Figure 4.10 B) and ERβ (Figure 4.10 C) mRNA levels was observed.

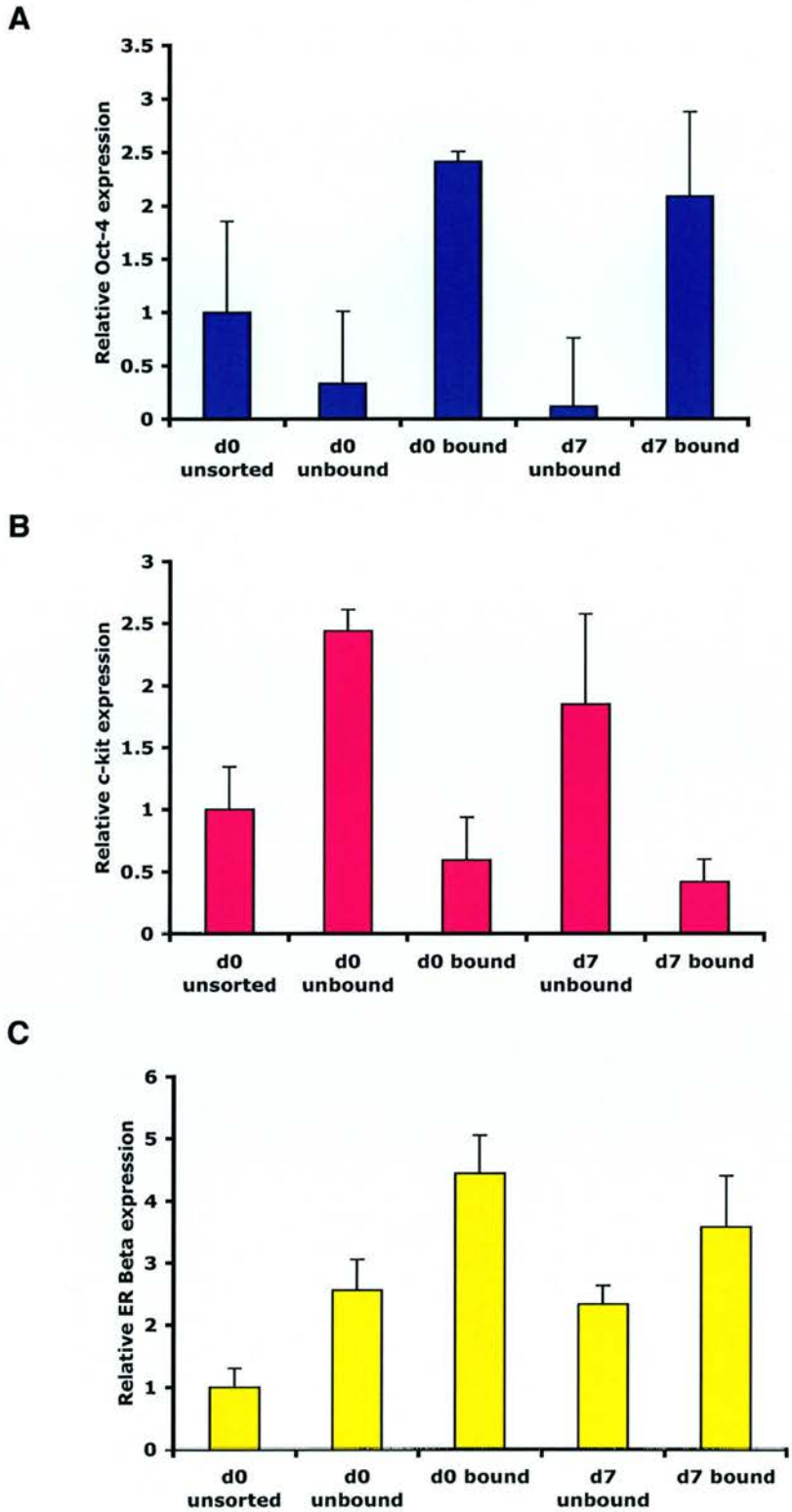


Figure 4.10 Effect of culturing the spermatogonial cells for 7 days on the relative levels of expression of Oct-4 (A), c-kit (B) and ERβ (C) in sorted, bound cell populations at d0 and d7 compared to an unsorted d0 population. Results are ± SEM n=3. Statistical analysis was performed by the use of one-way ANOVA.

4.3.6. Impact of ligands, growth factors and feeder cells on the differentiation status of the spermatogonial stem cell

The impact of ligands, growth factors and feeder cells that have previously been suggested to play a role in the differentiation of stem cells was investigated using the immunobead sorted cell populations. Cells were either cultured with LIF, SCF or E_2 alone or on top of a feeder layer of mitotically inactivated STO cells for 7 days prior to RNA analysis and Taqman analysis.

4.3.6.1. LIF

Treatment with LIF, which has been shown to maintain stem cells in an undifferentiated state, had no statistically significant effect on Oct-4 mRNA expression in the sorted, bound cell population showing compared to an untreated, sorted cell population (Figure 4.11 A). No change was observed in c-kit levels or ERβ mRNA levels (Figure 4.11 B and C) compared to the untreated sorted cell population.

4.3.6.2. SCF

Culturing the sorted, bound population of cells for 7 days in the presence of SCF had no effect on the expression of Oct-4 mRNA compared to pre-treatment cells (Figure 4.12 A). Treatment with SCF also resulted no statistically significant difference in both c-kit and ERβ mRNA expression (Figure 4.12 B and C) as shown by Taqman quantitative RT-PCR.

4.3.6.3. 17β oestradiol

The effect of the exposure to 17β oestradiol on the differentiation status of the bound cell population was investigated. The relative amounts of Oct-4, c-kit and ERβ mRNA were not significantly different after treatment with E_2 (Figure 4.13 A, B and C).

4.3.6.4. Co-culture with STO cells

After isolation and immunomagnetic sorting (section 4.2.2), the bound population were cultured for 7 days on mitomycin–c treated STO cells (section 4.2.7.1). The STO cells do not divide due to the mitomycin-c treatment and form a monolayer for the cells to sit upon. After the culture period, RNA was extracted from the cells and Taqman quantitative RT-PCR was performed and levels of Oct-4, c-kit and ERβ mRNA levels were also measured in were cells maintained in culture for 7 days without STO feeder cells and expression was compared to bound, uncultured population (Figure 4.14). Levels of Oct-4 (Figure 4.14.A), c-kit (Figure 4.14.B) and ERβ (Figure 4.14 C) mRNA were found to be unchanged in the cells cultured on the STO feeders.

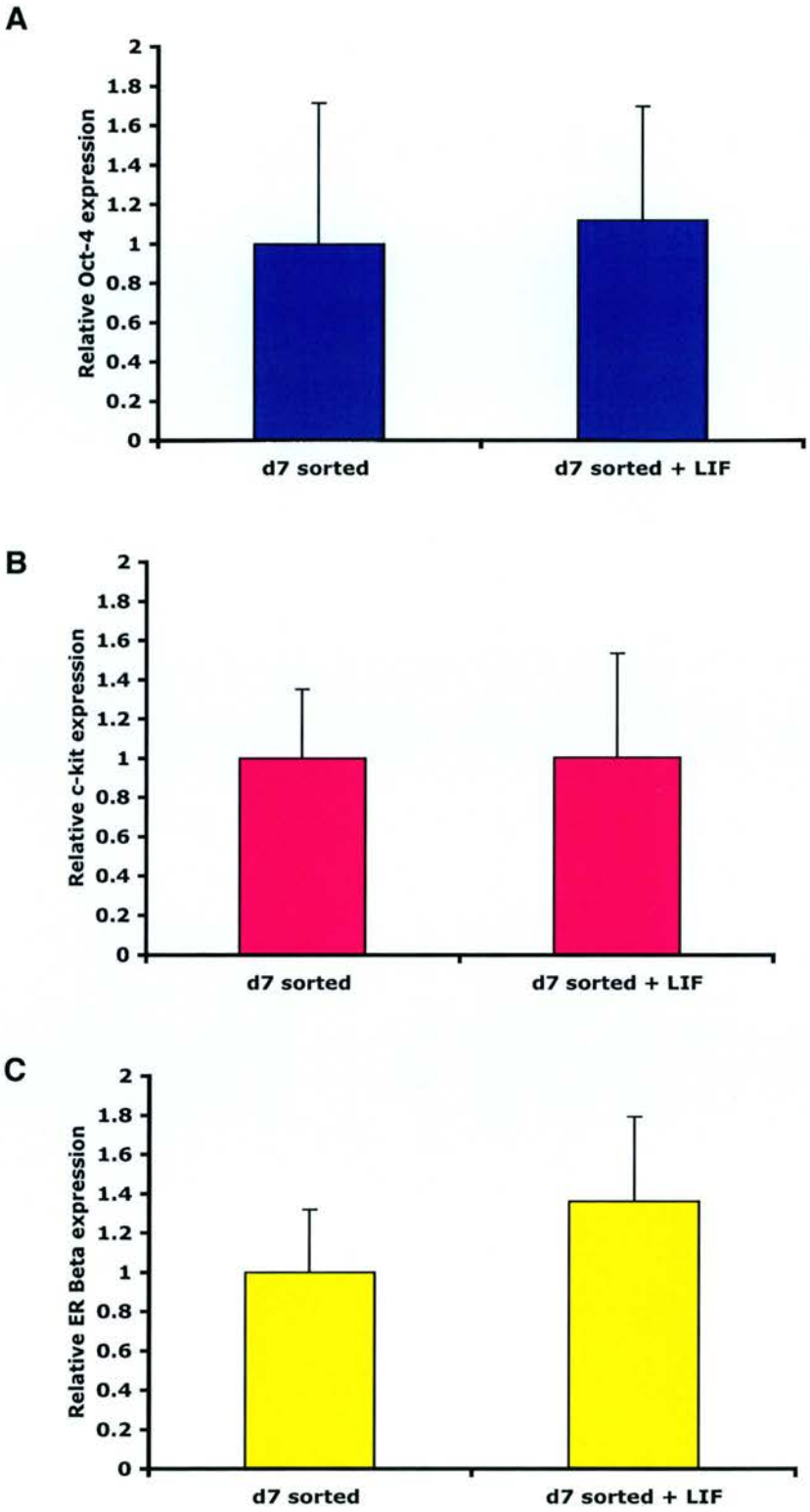


Figure 4.11 Effect of 7 days of LIF treatment in culture on the relative levels of Oct-4 (A), c-kit (B) and ERβ (C) on sorted, bound cell populations compared to a bound, untreated population. Results are ± SEM n=3. Statistical analysis was performed using a student's t-test.

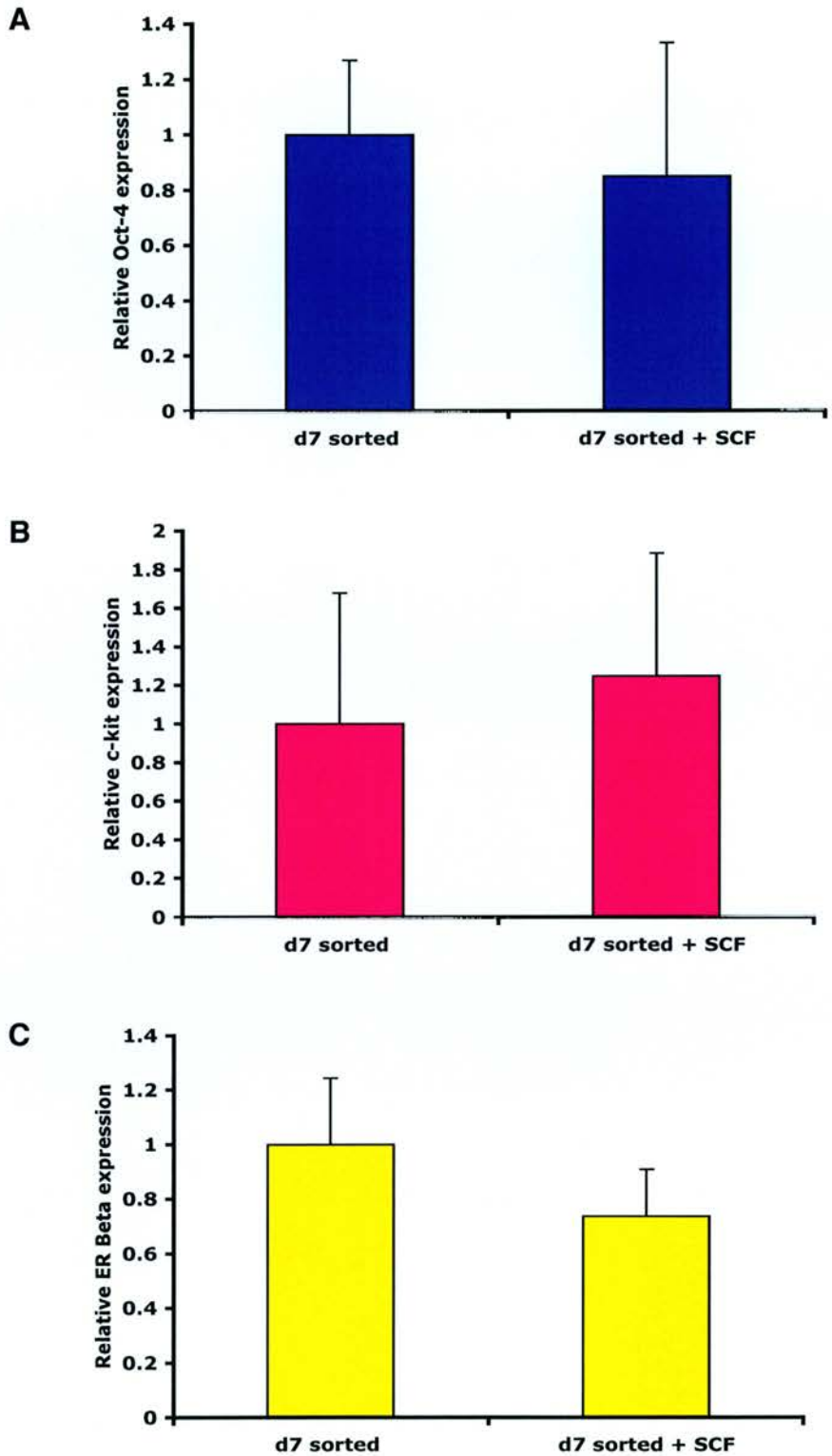


Figure 4.12 Effect of 7 days of SCF treatment in culture on the relative levels of Oct-4 (A), c-kit (B) and ERβ (C) on sorted, bound cell populations compared to a bound, untreated population. Results are ± SEM n=3. Statistical analysis was performed using a student's t-test.

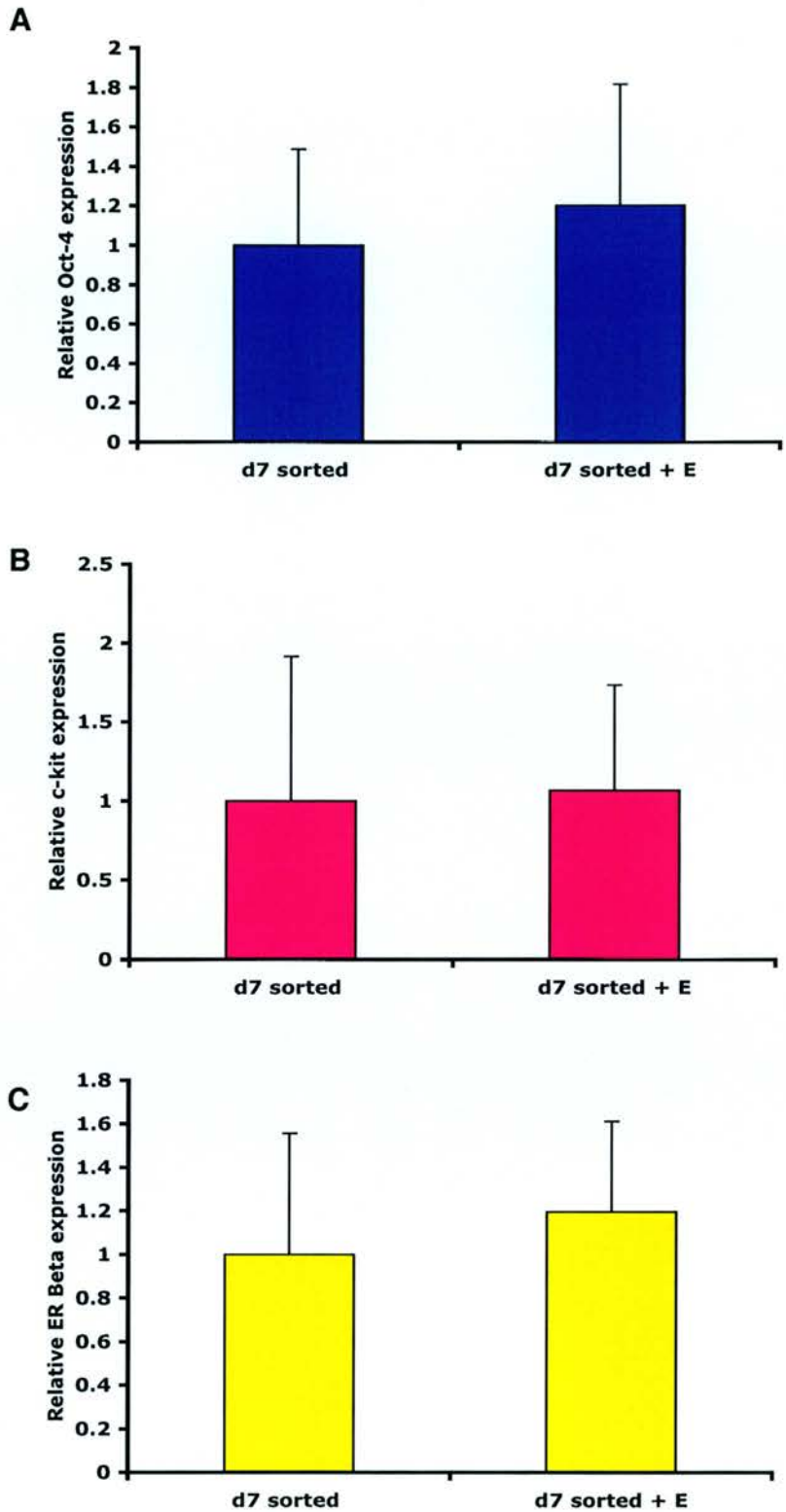


Figure 4.13 Effect of 7 days of 17β oestradiol treatment in culture on the relative levels of Oct-4 (A), c-kit (B) and ERβ (C) on sorted, bound cell populations compared to a bound, untreated population control. Results are ± SEM n=3. Statistical analysis was performed using a student's t-test.

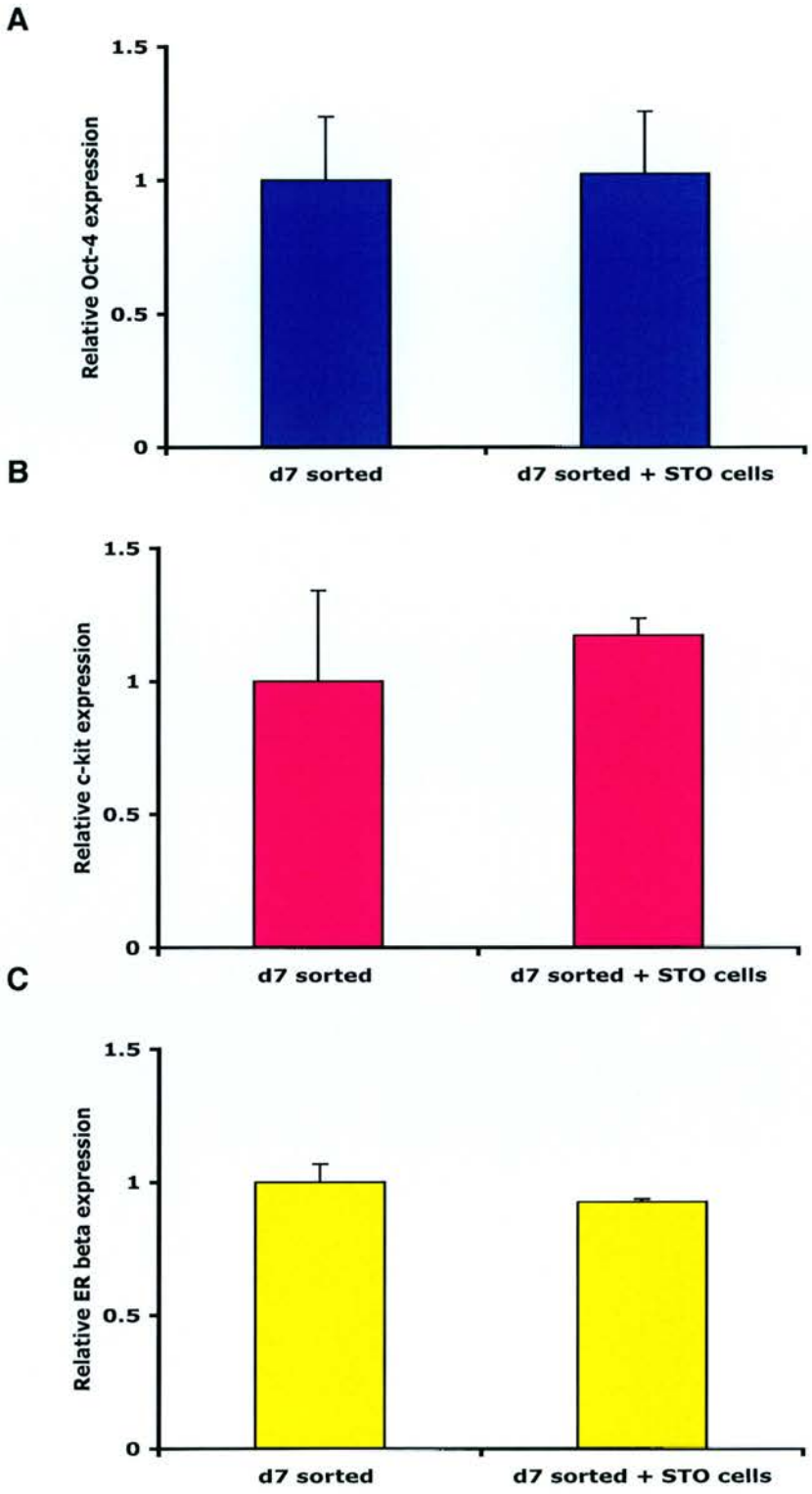


Figure 4.14 Effect of 7 days of culture with or without STO feeder cells on the relative levels of Oct-4 (A), c-kit (B) and ERβ (C) on sorted, bound cell populations compared to a uncultured, bound population. Results are ± SEM n=3. Statistical analysis was performed using a students t-test.

4.3.7. Impact of shRNA knockdown on the differentiation status of the spermatogonial stem cell

The experiments described in the previous sections (4.3.6) were designed to investigate whether the cells could be pushed into differentiating or proliferating in culture with the aid of various growth factors. As this was not achieved in this study, the effect of targeted depletion of Oct-4 or ERβ from the sorted cell population was assessed using shRNAs designed for Oct-4 and ERβ cloned into the pSilencer vector. Sequencing was performed to confirm the presence of the siOct-4 and the hairpin sequence (Figure 4.15). The siOct-4 sequence is underlined and shown in red. The hairpin sequence is shown in blue and the Bam H1/Hind III sites shown in green with a portion of the pSilencer sequence surrounding the insert shown in black. Sequencing revealed a G→C base change within the hairpin loop. As sequence specific knockdown is not dependant on the loop sequence, only the siRNA sequence, this error can be disregarded. The hairpin vector was introduced into immunomagnetic bead sorted spermatogonial stem cells using transfection using oligofectamine (Figure 4.16) or electroporation (Figure 4.17) 24 hours after cell isolation. Total RNA was recovered from the cells 2 or 6 days later (total of 3 and 7 days in culture) and levels compared to those of sorted cells not treated with shRNA. Relative expression of Oct-4, c-kit and ERβ as determined by Taqman quantitative RT-PCR established the effects of either Oct-4 or ERβ knockdown on the differentiation status of the cells.

4.3.7.1. Oct-4 shRNA

After 3 or 7 days in culture, Oct-4 mRNA levels were unchanged in the stem cell population transfected with the shOct-4 vector using oligofectamine compared to untransfected cells (Figure 4.16 A blue bars). No change in c-kit mRNA levels was observed in the transfected cells (Figure 4.16 A pink bars). Cells cultured with the shOct-4 vector did not show any alteration in Oct-4 mRNA (Figure 4.16 B, blue bars) or c-kit mRNA (Figure 4.16 B, pink bars).

After 3 or 7 days in culture, Oct-4 levels were successfully reduced in the stem cell population which were electroporated with the shOct-4 containing plasmid compared with un-electroporated cells (Figure 4.17, blue bars). Knockdown of Oct-4 was associated with an increase in c-kit mRNA in the cells (Figure 4.17 A, pink bars). The increase in the relative expression of c-kit in the sorted cells suggests that a proportion of the cells may have differentiated. After Oct-4 knockdown, ERβ mRNA expression was reduced compared to that of the untransfected spermatogonial stem cells (Figure 4 17 B).

TCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCG
CTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGT
TGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCC
AGTGATTCATATTTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAAC
GTGAATGTCTTTGGATTTGGGAATCTTATAAGTTCTGTATGAGACCACTC
GGATCCGTTTCTGAAGTGCCCGAAGTTCAAGAGACTTCGGGCACTTCA
GAAACTTTTTTGGAAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGT
GTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGC
ATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAA
TTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC

Figure 4.15 A shows the sequence of the shOct-4 hairpin containing pSilencer vector. The siOct-4 sense and antisense sequence are underlined and shown in red. The hairpin loop sequence is shown in blue with the BamH1/HindIII sites used to clone the hairpin insert shown in green. A portion of the pSilencer sequence at either side of the insert is shown in black. B shows how the hairpin folds up with the loop sequence shown in blue and the siERβ sense and antisense sequences shown in red. In both cases, the G→C base change within the loop sequence is shown in brown and underlined.

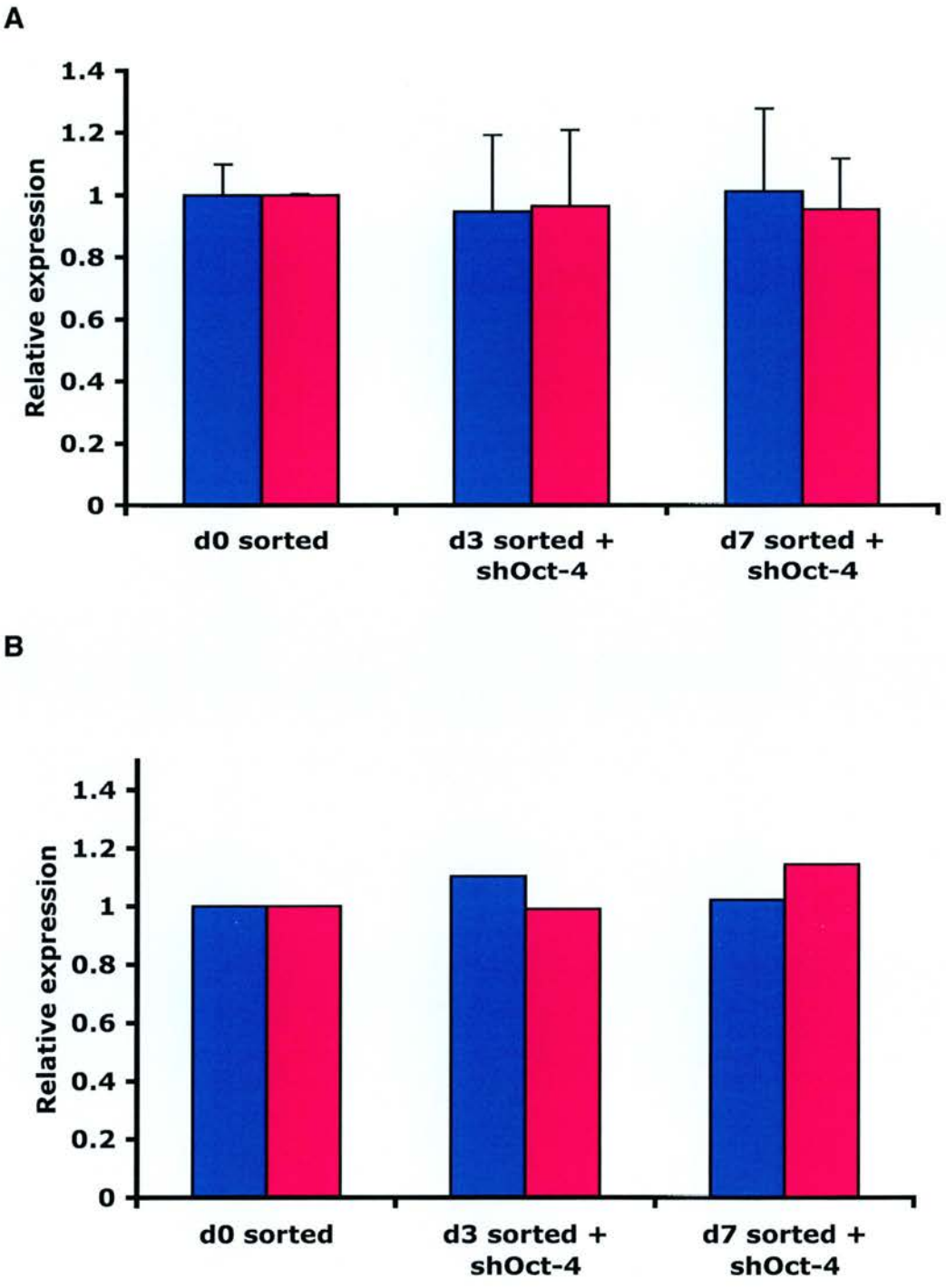


Figure 4.16 A shows the expression of Oct-4 (blue bars) and c-kit (pink bars) mRNAs in sorted spermatogonial stem cells after transfection with shOct-4 using oligofectamine after 3 and 7 days in culture compared to an untransfected sorted cell population Results are ± SEM n=3. B shows the expression of Oct-4 (blue bars) and c-kit (pink bars) mRNAs in sorted spermatogonial stem cells after culture with shOct-4 after 3 and 7 days in culture compared to an untreated sorted cell population Results are n=1. Statistical analysis was performed by the use of one-way ANOVA. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.001$.

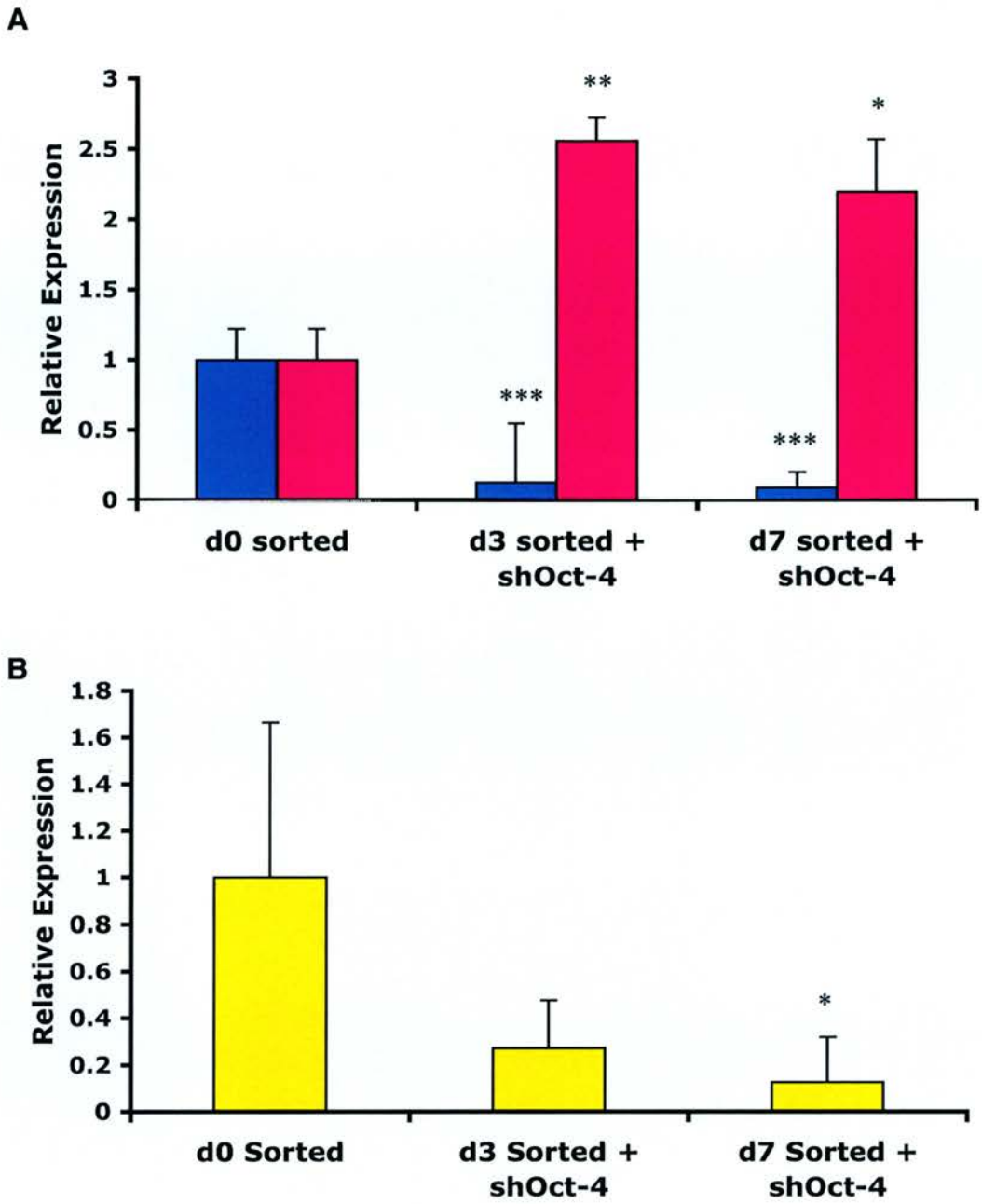


Figure 4.17 Panel A shows the expression of Oct-4 (blue bars) and c-kit (pink bars) mRNAs in sorted spermatogonial stem cells after electroporation with shOct-4 after 3 and 7 days in culture (d3 and d7) compared to an un-electroporated sorted cell population. Panel B shows relative ERβ mRNA expression at d3 and d7 in the same cells after Oct-4 knockdown compared to an untransfected sorted cell population. Results are ± SEM n=3. Statistical analysis was performed by the use of one-way ANOVA. * $P < 0.05$; ** $P < 0.01$: *** $P < 0.001$.

4.3.7.2. ERβ shRNA

As ERβ was shown to be expressed in the stem cell population (section 4.3.4.3), the effect of knocking down ERβ levels within the sorted cell population was assessed using an shRNA designed for ERβ. The hairpin vector was introduced into the cells using electroporation. ERβ mRNA levels were reduced in the sorted cells treated with shERβ both 3 and 7 days after electroporation (Figure 4.18), compared to a sorted cell population which were not electroporated. Expression of Oct-4 (Figure 4.19 B, blue bars) and c-kit levels (Figure 4.19 B pink bars) in the same cells after electroporation with shERβ were unchanged compared un-electroporated cells.

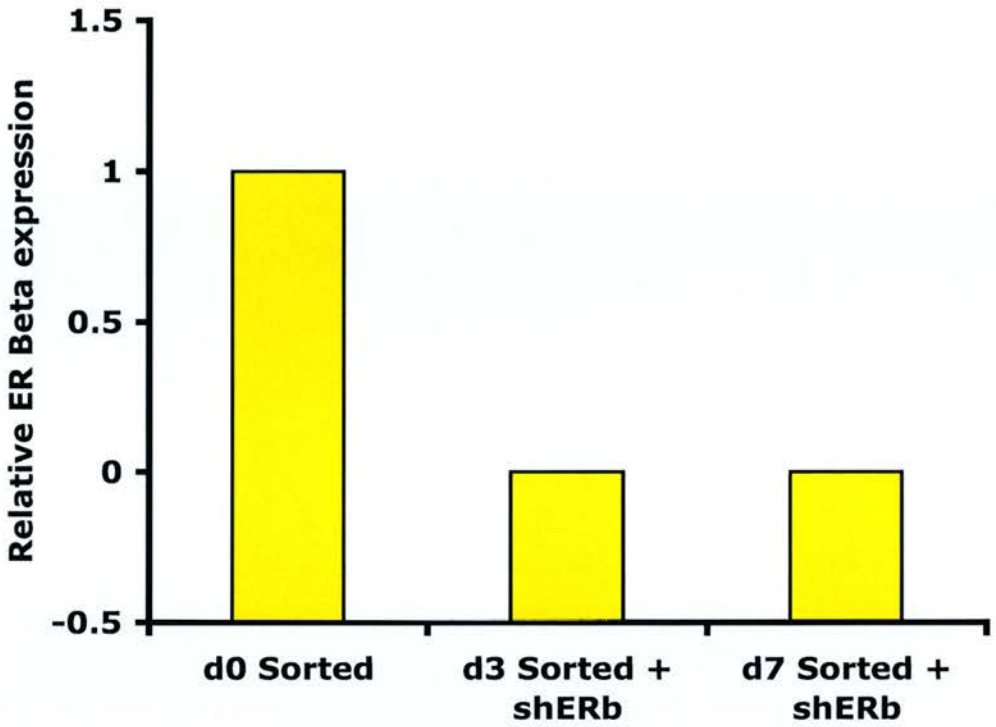


Figure 4.18 Reduction in ERβ mRNA levels after electroporation of sorted cell populations with shERβ, 3 and 7 days after treatment, n = 1.

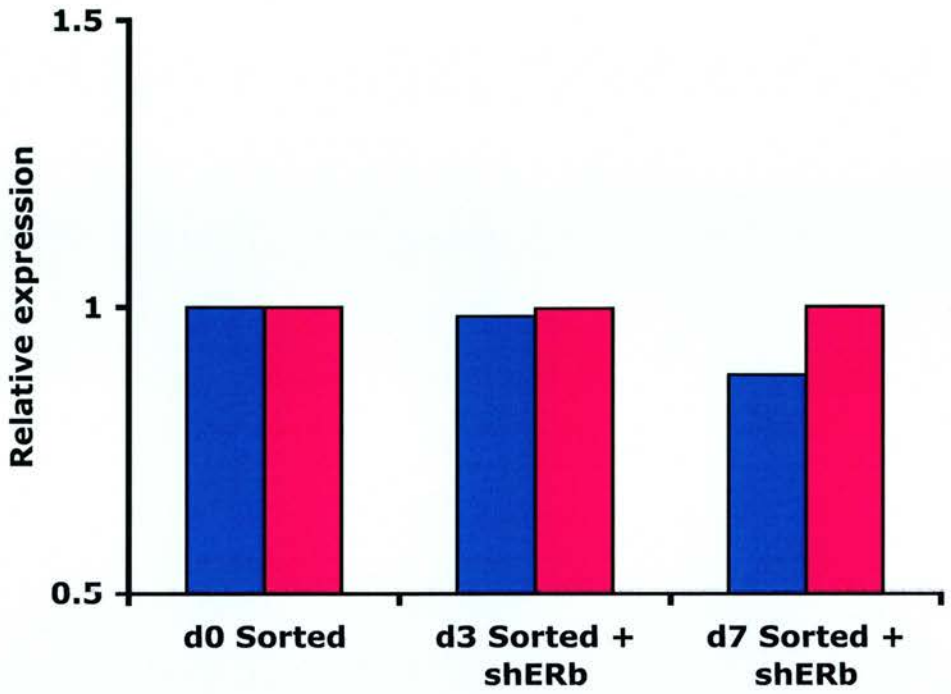


Figure 4.19 Oct-4 (blue bars) and c-kit (pink bars) mRNA levels in the sorted cell population after electroporation with an shERβ vector, n = 1.

4.4. Discussion

Stem cells of different organs share many characteristics. For example, they usually reside on the basement membrane and continue to self renew throughout adult life (Spradling et al., 2001). Spermatogonial cell lines could greatly facilitate research on spermatogonial stem cells, enabling analysis of mRNA and protein expression and would allow investigations into mechanisms that regulate stem cell proliferation and differentiation. Initial studies in this chapter used two germ cell lines, (GC-1 and GC-2); the preparation of which was originally described about 10 years ago (Hofmann et al., 1994; Hofmann et al., 1992). The GC-1 cells, when cultured alongside immortalised Sertoli cells, Leydig cells and peritubular myoid cells, were reported to form structures that resembled seminiferous tubules with the GC-1 cells aggregating in the centre of the "tubule" with the Sertoli cells (Hofmann et al., 1992). The GC-2 cell line, are under the control of the temperature sensitive SV40 large-T antigen and when cultured at the permissive temperature, the cells were reported to undergo meiosis *in vitro* (Hofmann et al., 1994). This was demonstrated by the use of flow cytometry, which showed a change in the cells from being a diploid population to a cell line with a haploid DNA content (Hofmann et al., 1994). However only two years later, it was discovered that over time, the cells had de-differentiated and the haploid phenotype of the cells was no longer seen. In the present study RT-PCR analysis of both cell lines did not result in detection of any of the germ cell markers previously identified in the cells (Wolkowicz et al., 1996). Furthermore, semi-quantitative RT-PCR revealed that the GC-1 and GC-2 cells did not express mRNA for the stem cell marker Oct-4. This result was not unexpected as the GC-1 cells were derived from B–type spermatogonia and spermatocytes and the GC-2 cells were reported to resemble spermatids. When the GC-1 cells were first isolated they were reported to express alkaline phosphatase mRNA (Hofmann et al., 1992), which is associated with germ cells (McCoshen and McCallion, 1975).

Although the results obtained confirmed that neither cell line expressed the expected range of germ cell markers (e.g. c-kit, Dazl, rbm), the cells may still provide a useful tool. For example, the cells did not express ERβ and therefore represent a testicular

cell line that was devoid of steroid receptors, which could be used for transfection studies. Unfortunately, preliminary transfection studies using the GC-1 cells failed to result in steroid responsiveness (not shown). This could have been due to problems in the transfection procedure or because the cells did not contain the necessary machinery to exert a response. The studies with this cell line were discontinued.

Following the preliminary work using the immortalised germ cells cell lines, further investigations focussed on using spermatogonial stem cells which were freshly isolated from the mouse testis. Testes from 8-day-old animals were used as the source of germ cells for these studies because mice of this age have a higher percentage of undifferentiated spermatogonia than adult animals. Analysis of 8 day old testes using immunohistochemistry and RT-PCR detected the expression of both the stem cell marker Oct-4 and the differentiated spermatogonial marker c-kit.

Studies carried out by Brinster and colleagues using germ cell transplantation methods have demonstrated that the efficiency of the technique was improved when cells were pre-selected using antibodies directed against the surface markers α-6 and β1 integrin (Shinohara et al., 1999). As the only cells that will repopulate the testes after germ cell transplantation are the spermatogonial stem cells, this data was taken as evidence that these proteins were expressed on the surface of these cells. In the present study taking this information into account, magnetic bead sorting was carried out using anti α-6-integrin and anti GFRα-1 antibodies and the germ cell population bound to the magnetic beads was characterised. The efficiency of both antibodies was comparable so it was decided to conduct all subsequent experiments using the anti α-6-integrin antibody

Semi-quantitative RT-PCR was not sensitive enough to pick up changes in the mRNA levels within the sorted cell populations so quantitative RT-PCR was performed by TaqMan. After immunomagnetic bead isolation, the bound population of cells, presumed to contain the stem cell population, was analysed and the presence of mRNA for the stem cell marker Oct-4 was confirmed. The amount of Oct-4

mRNA was higher in this population than in the unbound population showing evidence of successful isolation of the cells. The unbound population was assumed to contain differentiated spermatogonia. Expression of c-kit mRNA within the putative stem cell population was negligible and c-kit expression in the unbound population was increased 8 fold compared to the pre-sorted population of cells. These results confirm that cell sorting using immunomagnetic beads is an effective way of isolating the Oct-4 positive, spermatogonial stem cell population. Furthermore, c-kit mRNA was very low in this population of cells, which is in agreement with the studies by Schrans-Stassen et al. (1999) and Shinohara et al. (1999) that reported spermatogonial stem cells do not express c-kit.

The expression of ERβ mRNA and protein has been reported to occur in the testicular germ cells of rodents, primates and the human (Saunders et al., 1998; Saunders et al., 2001; Zhou et al., 2002) however, it was not clear if the spermatogonial stem cell population expressed ERβ. In the present study, the Oct-4 positive cell population was also positive for ERβ mRNA and furthermore, the levels were higher than in the unbound cells. These results demonstrate for the first time that ERβ is expressed in the murine spermatogonial stem cell population. Further studies will be needed to establish if this is true of spermatogonial stem cells of other species.

Many studies have shown that spermatogonial stem cells can be cultured for long periods of time (Brinster and Nagano, 1998; Dirami et al., 1999; Kanatsu-Shinohara et al., 2003; Nagano et al., 1998; Nagano et al., 2003; Parks et al., 2003). However, attempts to make the cells proliferate have met with limited success suggesting that either the proliferation rate of the stem cell population is very slow or the culture conditions are suboptimal. Some of the studies described in this chapter have used growth factors and feeder cells to investigate whether the differentiation status of the cells could be manipulated. Previous studies have used similar approaches to manipulate murine ES cells (Evans and Kaufman, 1981; Williams et al., 1988).

In the present studies, before experiments were carried out investigating the effects of growth factors, the expression of Oct-4, c-kit and ERβ mRNAs were measured in the cells both before and after culture for 3 or 7 days in medium alone. No differences in the levels of expression were observed suggesting that the culture system per se did not affect cell function.

The two growth factors chosen for study were LIF and SCF. LIF is commonly used to maintain mouse embryonic stem cells in an undifferentiated state (Murray and Edgar, 2001; Williams et al., 1988). Culture on a feeder layer of fibroblast cells also achieves the same result, with the removal of feeder cells or LIF from cultures resulting in differentiation of the cells (Evans and Kaufman, 1981). SCF is a Sertoli cell-produced paracrine regulator and acts as a survival factor for spermatogonia in the rat and pig (Dirami et al., 1999; Hakovirta et al., 1999). It has been shown that the spermatogonial stem cell proliferation can be significantly enhanced by the addition of SCF at 30 ng/ml (Yin and Li, 2002), furthermore, SCF has been shown to stimulate DNA synthesis in spermatogonial cells (Rossi et al., 1993). In the newt, recombinant human SCF was found to stimulate spermatogonial proliferation, but not the initiation of meiosis (Abe et al., 2002).

Addition of LIF to the sorted, bound cells did not result in any change in the expression of Oct-4, c-kit or ERβ mRNA suggesting that LIF does not alter the status of the stem cell population. This finding would be consistent with maintenance of stem cell characteristics by LIF which has been shown for ES cells (Williams et al., 1988). Addition of SCF to the cultured cells resulted no change in Oct-4 mRNA expression. Expression of ERβ mRNA was also unaltered. Although not significant, slight changes in mRNA expression were observed and more studies will have to be performed in order to increase n numbers to deterime whether a statistically significant difference can be observed to assess if cells are differentiating. A study performed by Godin et al. in 1991 using an in vitro assay system, showed that treatment of PGCs isolated from wild-type mouse embryos with SCF increased the number of PGCs (Godin et al., 1991) so it is also possible that the increase in c-kit

levels is due to an overall increase in cell number. This aspect could be investigated using cell proliferation studies. As well as supporting the maintenance of stem cells, additional roles for LIF and SCF have been proposed. The study by Pesce et al., in 1993 reported that treatment with LIF or SCF prevented proliferating PGCs from undergoing apoptosis in vitro. It is speculated that failure of this apoptotic event in vivo could result in an accumulation of aberrant survival of germ cells, which in turn could lead to the development of teratocarcinoma stem cells (Pesce et al., 1993). This information could be important in investigations into the causes of germ cell cancers in the testis, which are believed to be associated with problems in the differentiation and proliferation of spermatogonial stem cells.

Oestrogens have been shown to inhibit the development of gonocytes and Leydig and Sertoli cells in fetal rat testis *in vitro* (Lassurguere et al., 2003). However, other studies using isolated neonatal gonocytes, have reported that oestrogens can have a positive effect on gonocytes development (Li et al., 1997). The studies carried out by Atanassova et al. in 2000 in the rat have provided evidence that although high doses of potent oestrogens, such as DES, are inhibitory to testicular development, and treatment with low doses result in a stimulatory effect (Atanassova et al., 2000). It has been also been shown that 17β oestradiol can stimulate spermatogonial stem cell renewal in the Japanese eel (Miura et al., 1999). It is with this background, combined with the evidence presented in this thesis that ERβ mRNA is expressed in the spermatogonial stem cell population in the prepubertal testis that the effect of adding 17β oestradiol to cultured murine spermatogonial stem cells was investigated. In these experiments, although amounts of Oct-4 and ERβ mRNA were slightly increased by addition of 17β oestradiol, this was not statistically significant. No change was observed in c-kit mRNA expression suggesting that oestrogen exposure did not induce stem cells to differentiate and increases in mRNAs may therefore be due to an increased numbers of cells. Further studies are required to determine if this is the cause.

Murine and human ES cells are commonly grown on a feeder layer of fibroblast cells. These cells secrete factors that maintain the undifferentiated phenotype of the cells. Culturing the cells on a feeder layer of STO fibroblasts had no effect on the relative expression of Oct-4, c-kit or ERβ and therefore had no effect on the differentiation status of the cells. It has been proposed that LIF is one of the factors secreted by feeder fibroblasts as mouse ES cells grown on feeders do not require exogenous LIF in order to remain undifferentiated. However, totipotentiality of human ES cells does not depend totally on LIF (Thomson et al., 1998) and it has also been shown that maintenance of totipotency in human ES cells is STAT3 independent (Humphrey et al., 2004) indicating that in the human, LIF is not involved in the maintenance of undifferentiated cells suggesting that other factors secreted by the feeder cells may contribute to their maintenance. Similar findings have been reported in the primate where it has been shown that the LIF/STAT3 pathway functions in embryonic stem cells made from cynomolgus monkeys but this system is not essential for the maintenance of self-renewal. These studies together suggest that the primate ES cells are also maintained in an undifferentiated state through LIF/STAT3-independent signalling (Sumi et al., 2004).

The study by Velkey and O'Shea in 2003 demonstrated that in vitro differentiation of murine ES cells could be induced by RNA interference. They showed that when Oct-4 expression was knocked down in the cells using a short hairpin RNA specific to Oct-4, this triggered differentiation of the cells containing the shRNA into trophectoderm cells (Velkey and O'Shea, 2003). In the current study, knockdown of Oct-4 mRNA using a plasmid vector containing an shRNA based on the one published by Velkey and O'Shea was carried out on the using the putative spermatogonial Oct-4 positive stem cell population. After the vector was constructed, a single base change was detected in the hairpin loop section of the insert. As this part of the hairpin should have no effect on gene silencing (only the sequence specific Oct-4 19mer sequence causes knockdown), it was decided to proceed with this vector. Transfection of the cells using oligofectamine, a standard transfection reagent, had no effect on the levels of Oct-4 mRNA in the cells. Incubating the cells with the shRNA alone also had no effect on Oct-4 mRNA expression. However, after

electoporation, a reduction in the expression of Oct-4 mRNA was observed at both time points (3 and 7 days). In the same cells, this was accompanied by an increase in the expression of c-kit mRNA showing that by reducing the expression of Oct-4, cells in the stem cell population had entered a differentiation pathway. When the expression of ERβ mRNA was investigated, it was shown that at both time points, a reduction in Oct-4 was accompanied by a reduction in ERβ mRNA. This result supports the finding that the Oct-4 positive stem cell population express higher levels of ERβ mRNA than the c-kit positive differentiated spermatogonial population. Further experiments could be carried out to try and block Oct-4 knockdown induced cell differentiation using LIF or other compounds. The molecular mechanisms necessary for maintenance of self-renewal in response to LIF signalling have been investigated using murine ES cells. To date, Stat3 and Nanog (Chambers et al., 2003; Mitsui et al., 2003) together with Oct-4 seem to play key roles (Niwa, 2001). Further studies using siRNA to independently manipulate levels of expression of Nanog and STAT3 in spermatogonial stem cells are planned.

After electroporation with the shERβ containing vector, ERβ levels in the sorted cell population were reduced showing that the hairpin vector was causing sequence specific knockdown of receptor levels. The reduction in ERβ had no effect on the levels of Oct-4 or c-kit suggesting that ERβ, although present in the stem cell population, does not play a role in the regulation of differentiation. What role ERβ plays in the spermatogonial stem cell remains unknown and further studies are planned to investigate this.

In conclusion, the studies performed in this chapter have shown that the spermatogonial stem cell population can be isolated and successfully enriched using immunomagnetic beads. This population, which has been shown for the first time to be ERβ positive can be manipulated using RNA interference and can be induced to enter a differentiation pathway by selectively reducing expression of Oct-4.

Chapter 5

General Discussion

5.1. Introduction

Spermatogenesis, the process whereby diploid spermatogonial stem cells divide and differentiate to become mature spermatozoa is a precisely regulated process in which the germ cells closely interact with the somatic Sertoli cells of the testis. These interactions between germ cells and Sertoli cells play a major role in the establishment and maintenance of spermatogenesis and fertility in the male. Steroid hormones regulate cell function via receptors, which are expressed within their target tissues. Both oestrogen receptors (α and β) and the androgen receptor (AR) have been immunolocalised to the male reproductive tract of many species. Sertoli cells express both ERβ and AR while murine germ cells only express ERβ (Bremner et al., 1994; McKinnell et al., 2001; Nie et al., 2002; Saunders et al., 1998; Saunders et al., 2001; Zhou et al., 2002).

5.2. Role of steroid hormones in spermatogenesis

The generation of knockout mice for the steroid hormone receptors and the enzymes responsible for steroid synthesis gave new insight to how these function within the reproductive system (Couse et al., 1999; Dupont et al., 2000; Eddy et al., 1996; Fisher et al., 1998; Krege et al., 1998; Lubahn et al., 1993; Yeh et al., 2002). In the case of the oestrogen receptor knockout animals, male ERα knockout mice (ERKO) are infertile and have major abnormalities in the seminiferous epithelium. ERα is expressed in high levels in the efferent ducts of most species and the knockouts exhibit atrophic testes caused by abnormal fluid reabsorbtion in the efferent ductules resulting in impaired spermatogenesis (Lubahn et al., 1993). Male ERβ knockout mice (βERKO) (Dupont et al., 2000; Krege et al., 1998) are fertile and show no major alterations in reproductive tract function (Couse and Korach, 1999). In humans, one ERα deficient patient has been described: this patient had a normal sperm count but the viability of the sperm was reduced (Smith et al., 1994). To date

no individuals lacking ERβ have been identified although polymorphisms in the ERβ gene have been associated with some fertility problems in women as well as problems in the bone and with blood pressure (Ogawa et al., 2000a; Ogawa et al., 2000b; Sundarrajan et al., 2001). Studies using the double knockout ERαβKO mice have suggested that the primary role of ERβ is to modulate gene regulation via ERα (Weihua et al., 2000). Studies in the hpg mouse have suggested that treatment with oestrogen is sufficient to restore spermatogenesis (Ebling et al., 2000). A number of patients with defects in the aromatase gene have been identified and mouse models with a targeted deletion of the *cyp 19* gene created (Fisher et al., 1998; Murata et al., 2002; Robertson et al., 2001; Toda et al., 2001). Notably, ArKO male mice, which lack the ability to synthesise oestrogen, become infertile and show an increase in levels of apoptosis in round spermatids. The phenotype of these mice is in marked contrast to the double ERKO/βERKO male mice, which have a phenotype resembling the ERKO male (showing dilation of the seminiferous tubules, and ovaries in which the granulosa cells develop characteristics of Sertoli cells (Couse et al., 1999; Dupont et al., 2000)). Excess amounts of oestrogens, occur in the EST knockout mice; these animals suffer from a progressive and age dependant reduction in fertility caused by unmetabolised oestrogen (Qian et al., 2001; Tong and Song, 2002). However, given the variation in the results obtained with the different murine models and the gross disturbance in the levels of other hormones (gonadotrophins and androgens) seen in some cases, questions remain as to the exact role of oestrogens in the regulation of spermatogenesis.

In humans, the absence of the X-linked AR gene results in androgen insensitivity syndrome, this condition describes a range of phenotypic abnormalities of male sexual development in humans, which are described fully in the review by Quigley (1995). Male patients present with predominantly female phenotypic development and are infertile (Quigley et al., 1995). Mice with naturally occurring mutations in AR (Charest et al., 1991; Murphy et al., 1994) as well as those in which complete ablation of AR has been induced by cross-breeding mice containing a floxed AR allele with mice expressing CRE recombinase under the control of a ubiquitous

promoter (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004) have been described. In both cases, germ cell development had been impaired and very few haploid cells were found. The mice developed an abnormal urogenital tract with the testis failing to descend into the scrotum and the mice were infertile. Because cryptorchidism in mice results in impaired spermatogenesis associated with meiotic arrest of the germ cells, it is unclear whether the loss of the androgen receptor is causing the defect in spermatogenesis or if it merely a consequence of the cryptorchidism (Holdcraft and Braun, 2004). The results obtained from the study of the selective Sertoli cell knockouts clearly show that it is the loss of AR that causes fertility problems in these animals; the testis are correctly positioned in the testis but still show impaired spermatogenesis (Chang et al., 2004; De Gendt et al., 2004). These findings demonstrate a clear role for AR expression in Sertoli cells influencing germ cell function in adulthood. Whether local oestrogen biosynthesis and/or expression of ERβ in testicular cells play a role in male fertility remains a topic for debate as yet no cell selective knockouts of ERβ have been attempted.

5.3. Findings of this thesis

This studies described in this thesis set out to investigate the role of steroid hormones, in particular oestrogens, in modulating the function of germ cells and the somatic cells of the testis in an attempt to achieve better understanding of how oestrogens might contribute to the process of spermatogenesis and male fertility. During the course of the studies presented in this thesis, techniques for the application of RNA interference in mammalian systems were first described (Elbashir et al., 2001). As this presented a way to actively manipulate gene expression in the cells under study, this technique was utilised and has proved to be very useful in the current studies for reducing the levels of specific genes in both germ cells and Sertoli cells.

In Chapter 3, studies focussed on the role of steroid hormones in Sertoli cell function. The studies in this chapter were carried out using the SK11 cell line (Walther et al., 1996), and the results obtained highlighted the usefulness of this cell line in the study of the Sertoli cell *in vitro*. One of the advantages of using these cells

is that they can be maintained in both an undifferentiated and differentiated state due to the presence of the SV40 large T antigen. Initial characterisation of the cells showed that although they had been in use in our laboratory for several years, they still expressed many proteins identified in Sertoli cells in vivo including both ERβ and AR. The expression of both receptors in these cells made it possible to conduct studies in which the steroid responsiveness of the cells was tested. Androgen stimulation led to an increase in AR mRNA as shown by TaqMan RT-PCR. Similarly, stimulation with 17β oestradiol led to an increase in ERβ mRNA. Reporter constructs containing EREs or AREs were activated following addition of steroid ligands in the cells. Therefore a key finding from the current experiments was that not only did the SK11 cells contain a functional ERβ and AR, but also that one consequence of ligand stimulation was a direct up-regulation of their respective receptor genes. Further studies are required to see if this also occurs *in vivo* but if it does, it could have consequences for the impact of steroids on testicular function.

RNA interference by means of a linear siRNA, as well as an shRNA delivered in a plasmid vector, was effective in inducing target selective knockdown of ERβ in the Sertoli cells. The increase in expression of ERß mRNA that is usually observed following exposure of cells to oestradiol was blocked and no activation of the ERE containing reporter was observed. Attempts to perform similar experiments with the androgen receptor failed, as an effective siAR sequence was not found in the course of these investigations. These findings illustrate that although RNA interference can be an effective technique, currently there are limitations in our ability to pick the correct sequence for targeting with 100% success. New algorithms are being tested and better web based design tools have already become available so it is likely that a suitable sequence to target AR will be found.

In addition, silencing gene expression by RNAi requires efficient delivery of siRNAs into cells and this can be particularly problematic when primary cells or tissue fragments are being used. New methods focussing on delivery of shRNAs in viral vectors *in vitro* and *in vivo* (Barton and Medzhitov, 2002; Devroe and Silver, 2002;

Tiscornia et al., 2003) have recently been described. In the context of the current studies it is notable that injection of adenoviral vectors directly into the testis results in a preferential uptake in somatic cells (Blanchard and Boekelheide, 1997; Scobey et al., 2001). Additional studies using adenoviral vectors have confirmed that the route of injection has an effect on the uptake of the virus. After an intratubular injection, adenoviral transgene expression was observed in the Sertoli cells, however, after intratesticular injection, adenoviral expression was observed only in the Leydig cells (Kojima et al., 2003). No germ cell expression of the adenovirus was observed in either study demonstrating that delivery of an shRNA in an adenoviral vector could be a useful way to directly target gene expression in the somatic cells of the testis.

A further example of how viral vectors can be used to target Sertoli cells has been reported using the *Sl* (*Steel*, Kit-ligand deficient) mutant mouse, which is infertile due to defective Sertoli cells. In this case, an adenoviral vector carrying the *Sl* gene was able to partially rescue spermatogenesis resulting in production of round spermatids and spermatozoa in the seminiferous tubules. After intracytoplasmic sperm injection (ICSI), normal fertile offspring were produced. (Kanatsu-Shinohara et al., 2002). Importantly, the offspring did not show any germline transmission of the adenoviral DNA giving evidence that adenovirus-mediated gene transfer has a minimal risk for male germline transmission which is a factor to consider if these treatments were ever to be transferred to a human clinical situation. A similar study was carried out investigating restoration of kit ligand in Sertoli cells using lentiviral gene transfer (Ikawa et al., 2002). Again, restoration of spermatogenesis was observed and after ICSI, none of the offspring carried the lentiviral transgene. This opens up the possibility that introduction of shRNA via a lentiviral vector could offer an alternative to the adenoviral method.

Using this information and the results presented in this thesis, adenoviral mediated delivery of siRNA seems to be an exciting way to both target and knockdown expression of ERβ or AR in the Sertoli cells of the testis, *in vivo*. By knocking down each receptor in turn, investigations into what effect this would have on signalling

via the other receptor could be carried out. This approach could help us determine whether ER/AR crosstalk is occurring in the Sertoli cell and this will be the subject of future experiments.

Chapter 4 of this thesis focussed on the spermatogonial populations of the murine testis. Spermatogenesis depends on the ability of the subpopulation of spermatogonial stem cells to divide and to embark on the process of differentiation, which ultimately leads to the production of mature spermatozoa. The stem cell population must also remain stable in number throughout adult life. To date our ability to understand the process of germ cell development has been hindered by the limited availability of cells which can be studied in vitro. In the course of my studies I found that neither the GC-1 and GC-2 germ cell lines had maintained characteristics of the germ cells from which they were originally derived. The most promising report of a spermatogonial stem cell line came in 2002. The cells, which were immortalised using telomerase, were shown to maintain stem cell characteristics (Oct-4 expression, diploid phenotype) after many months in culture (Feng et al., 2002). However, to date, no transplantation studies have been carried out on these cells, which would be the true test of whether they have retained their totipotentiality and claims that they can be induced to differentiate in vitro have not been independently validated. In the fish, there has been a report of the derivation of a spermatogonial cell line from mature medakafish testis without immortalization. After 140 passages during 2 years of culture, this cell line retained a diploid phenotype and a gene expression pattern consistent with spermatogonial stem cells. The cells are stable but exhibit growth factor-dependent proliferation and the line has been shown to be capable of undergoing meiosis and spermiogenesis to generate motile sperm (Hong et al., 2004a; Hong et al., 2004b).

Isolation of an enriched population of spermatogonial stem cells was first shown after the discovery of the expression of the surface markers α-6 and β-1 integrin on the cells (Shinohara and Brinster, 2000; Shinohara et al., 2000). Using similar methods, in Chapter 4 of this thesis I was able to demonstrate enrichment of spermatogonial stem cells as shown by a higher proportion of Oct-4 positive cells, in

those bound to magnetic beads after incubation with antibodies directed against α6 and ß1 integrins. Using this population of cells, attempts were made to induce the stem cells to proliferate and differentiate in culture. The growth factors LIF and SCF were used but addition of these factors did not seem to have any effect on the cultured cells. The stem cell population was found to express ERβ mRNA. It has been shown in the past that ERβ mRNA and protein is expressed in the germ cells of the testis (Zhou et al., 2002) and exposure to oestrogens has been claimed to affect the function of gonocytes (Lassurguere et al., 2003; Li et al., 1997). Therefore the impact of 17β oestradiol on the stem cell population was investigated; a slight increase in expression of Oct-4 and ERβ mRNAs was observed but there was no effect on expression of c-kit levels suggesting that oestrogens alone are not able to induce spermatogonial stem cells to enter the differentiation pathway.

Culture of the stem cell population on feeder fibroblasts did not have any impact on Oct-4 or c-kit levels. A recent study using spermatogonia from rats expressing GFP exclusively in the germline showed that in specific culture conditions male germ cells lose, or maintain, stem cell activity depending on what type of feeder cells they are maintained upon. When cultured on STO cells, stem cell characteristics were lost however, when cultured on MSC-1 cells, a Sertoli cell line, the cells maintained stem cell activity as determined by flow cytometry (Kent Hamra et al., 2004). This information is not surprising as the close relationship between the Sertoli cell and the germ cell is well documented. It is possible that the stem cells require a factor produced by the Sertoli cells in order to proliferate and differentiate *in vitro*. Further work is proposed to look at this in the mouse, using the Sertoli cell line, SK11, utilised in this thesis.

After the success of using siRNA to knockdown gene expression in the Sertoli cells, the technique was used to investigate the effect of removing Oct-4 or ERβ on the pattern of gene expression in the enriched stem cell population. In 2003, a report showed that the differentiation status of murine ES cells could be altered using an shRNA directed against Oct-4 (Velkey and O'Shea, 2003). Utilising an shRNA based

on this sequence, Oct-4 mRNA expression was successfully knocked down in the stem cell population and this was shown to induce an increase in expression of c-kit mRNA, which is only expressed once spermatogonia have become committed to differentiation (Schrans-Stassen et al., 1999). In addition, as a complement to the experiments in which oestradiol was added to germ cell cultures, expression of ERß mRNA was knocked down with a specific shRNA. The consequent reduction in ERβ expression did not have any effect on the expression of Oct-4 mRNA, neither did it induce expression of c-kit. These results suggest that oestrogens acting via ERß are not involved in determining the differentiation status of the murine stem cells.

Although relatively little is known about what controls the maintenance/differentiation of the spermatogonial stem cell population, it is clear from my studies that Oct-4 is a key regulatory protein. Recently, studies on murine ES cells have led to the discovery of several new factors including Nanog, which has been reported to be involved in the relationship between Stat3 and LIF (Chambers et al., 2003; Mitsui et al., 2003) and the transcriptional repressor Plzf, which has been identified as a spermatogonia-specific transcription factor that is required to regulate self renewal and the maintenance of the stem cell population (Costoya et al., 2004). Nanog is expressed in the testis and together with Oct-4 is highly expressed in testicular germ cell tumours (Almstrup et al., 2004) therefore further studies manipulating levels of Nanog are planned as this may shed light on how this factor contributes to spermatogonial stem cell function.

Evidence that the delicate balance that exists between spermatogonial stem cell self renewal and differentiation must be maintained to ensure normal fertility has come from studies showing that testis degeneration can be induced by an increase in spermatogonial proliferation. One consequence of this is an increased entry of germ cells into meiosis and activation of meiotic checkpoints, which in turn can result in an increase in germ cell apoptosis (de Rooij and de Boer, 2003). Over the past fifty years, the incidence of testicular cancer has increased in a number of developed countries (Toppari et al., 1996) and increased exposure to natural and synthetic oestrogens has been cited as one potential cause of this increase (Sharpe and

Skakkebaek, 1993; Weir et al., 2000). These ideas have been supported by studies showing that high concentrations of oestrogens can stimulate PGC growth *in vitro* and this aberrant growth can result in the tumorigenic transformation of these cells (Moe-Behrens et al., 2003). This report did not investigate the expression of ERβ in the PGCs but suggests further studies on the impact of oestrogens on germ cell function are warranted.

5.4. Future studies

The studies presented in this thesis have shown that the SK11 cell line is a good system to study Sertoli cell function *in vitro*. The development of viral vectors expressing the shRNAs, thereby increasing the transfection efficiency and the levels of knockdown achieved in the cells, is planned. The transfer of the siRNA technology from *in vitro* studies in the SK11 cell line to *in vivo* experiments targeting the Sertoli cells is an exciting prospect which should yield interesting results.

Knockdown of androgen receptor expression in the SK11 cell line was attempted but a sequence that achieved good levels of knockdown was not obtained. Further work is required to design an efficient siRNA for AR to investigate the reduction of AR in the Sertoli cell, thereafter, *in vivo* studies allowing the acute ablation of Sertoli cell specific AR should provide a useful complement to the studies already underway with the SCARKO mice where loss of AR expression is life-long (De Gendt et al., 2004).

In comparison with the Sertoli cell, no suitable germ cell line was found. The results obtained by the use of primary spermatogonial stem cells has however been encouraging and future studies will focus on ways to induce proliferation of these stem cells. Notably it has been reported that addition of GDNF together with epidermal growth factor, basic fibroblast growth factor, and leukaemia inhibitory factor, was able to induce proliferation of murine gonocytes *in vitro* over a 5-month period 10^{14} fold. Furthermore, germ cell transplantation was used to show that the cells remained functionally active and were able to restore fertility to infertile recipient mice (Kanatsu-Shinohara et al., 2003). In the current studies, neither LIF,

SCF, nor oestradiol alone, had any effect on the proliferation or differentiation status of the isolated spermatogonia so further studies are necessary to establish whether addition of GDNF will influence cell survival/replication. Culture of murine ES cells on feeder fibroblast layers is a well-established technique. The presence of a feeder layer when the spermatogonial stem cells were cultured in this study did not have any effect. Reports have suggested that co-culture with Sertoli cells could influence spermatogonial stem cell proliferation and studies of this nature using the SK11 cell line are planned. Studies using RNAi mediated knockdown will be extended to investigate the role(s) of other genes such as Nanog (Chambers et al., 2003; Mitsui et al., 2003), which has been identified from studies in murine ES cells. This will add to the current understanding of the Stat3/Oct-4/LIF/Nanog relationship which is known to be important in the regulation of stem cell renewal in murine ES cells (Chambers et al., 2003; Mitsui et al., 2003).

If the cells can be induced to proliferate *in vitro* attempts will then be made to immortalise the stem cells. One report claiming to have achieved this using murine cells already exists (Feng et al., 2002), but doubts exist as to whether the cells have maintained an undifferentiated phenotype. It has however been achieved in other species, for example the medaka fish (Hong et al., 2004a; Hong et al., 2004b).

The role of ERβ in the stem cell population remains unknown. From these studies it seems likely that the role of ERβ is not in maintenance of the stem cell in an undifferentiated state but further studies are required to elucidate the role of ERβ in the spermatogonial stem cell.

5.5. Conclusion

Since the discovery that steroid hormones were produced in the testis (reviewed in Hess, 1997), much research has been undertaken to determine the role of steroid hormones, in particular oestrogens, in the male reproductive tract. The SK11 cell line has been shown to provide an excellent model for the investigation of the regulation of gene expression by steroid hormones and with the use of RNAi, manipulation of

gene expression has been made possible without the problems associated with generation of a knockout model.

These studies have shown for the first time that ERβ is expressed in the spermatogonial stem cell population. The use of RNAi in the spermatogonial stem cell population has resulted in alteration of the differentiation status of the cell and future studies will focus on the mechanism by which stem cell renewal and differentiation may be determined, and what role ERβ may play in this process.

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Appendix

Names and addresses of suppliers

Adobe Systems:	Mountainview, CA
Advanced Biotechnologies (Abgene):	Surrey, UK
Ambion:	Abingdon, UK
Amersham Life Sciences:	Buckinghamshire, UK
Berthold Technologies:	Hertfordshire, UK
BD Biosciences:	Oxford, UK
Bio-Rad Laboratories:	Hemel Hempstead, UK
Calbiochem:	Nottingham, UK
Cambridge Biosciences:	Cambridgeshire, UK
Cellstar ® Greiner bio-one:	Gloucestershire, UK
Clontech:	Hants, UK
Costar:	The Netherlands
DAKO Corp:	Cambridge, UK
Dynal:	Wirral, UK
Diagnostics Scotland:	Carluke, Scotland, UK
Eastman Kodak Co.:	Rochester, NY, USA
European Collection of Cell Culture (ECACC):	Wiltshire, UK.
Gene Therapy Systems (Cambridge Biosciences):	Cambridgeshire, UK
Genosys	Cambridgeshire, UK
Gibco:	Paisley, UK
GRI Syngene:	Essex, UK
Hoefer Scientific Instruments:	USA
Invitrogen:	The Netherlands
Kendro Laboratory:	Hertfordshire, UK
Leica:	Milton Keynes, UK
Millipore:	Watford, UK
MJ Reasearch:	MA, USA
MWG Biotech:	Wolverhampton, UK
Nalgene Nunc International:	Hereford, UK
New England Biosciences (NEB):	Hertfordshire, UK

Olympus Optical Co.:	London, UK
Pharmacia Biotech Qenequant:	Hertfordshire, UK
Photosol Ltd.:	Essex, UK
Plastik® Cultureware, MatTek Coporation:	MA, USA
PolyTransfection:	Sussex UK
Promega:	Southampton, UK
Q-Biogene:	UK
Qiagen:	West Sussex, UK
Roche:	East Sussex, UK
Sigma:	Poole, Dorset, UK
Stratagene:	Amsterdam,
Tefal:	Nottingham, UK
Tocris Cookson Ltd:	Bristol, UK
Vector SP-2001:	Peterborough, UK
VH Bio Ltd:	Newcastle, UK
Zeiss:	Hertfordshire, UK